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Comparison of Certain Properties of Human Plasminogen and "Proactivator". (23165)

FRANK B. ABLONDI AND JAMES J. HAGAN (Introduced by B. L. Hutchings)
(With the technical assistance of Ralph E. Clarke)
Biochemical Research Section, Research Division, American Cyanamid Company, Pearl River, N. Y.

Mammalian plasma contains an enzyme precursor termed plasminogen (profibrinolysin)(1) which can be activated by various agents *in vitro* to form plasmin (fibrinolysin)(1), an enzyme capable of dissolving fibrin clots. Streptokinase,* a commonly employed activator of human plasminogen, is incapable of activating some animal plasminogens(2). Geiger(3) first reported that more than one factor was involved in the activation of certain animal plasminogens by SK. Later, Müllertz and Lassen(4) showed that although bovine plasminogen was refractory to activation by SK, the addition of human globulin to the system resulted in the activation of the bovine plasminogen. As a result of their investigations, Müllertz and Lassen postulated that SK reacts with a proactivator (human factor) to form a plasminogen activator which converts plasminogen (bovine) to bovine plasmin. The studies of Troll *et al.*(5) and Troll and Sherry(6) indicated that this 2-stage activation process also applied to the SK activation of human plasminogen, the first step being a stoichiometric combination of SK with proactivator to form activator; the second stage, the enzymatic conversion of plasminogen to plasmin by this activator. Employing synthetic substrates, they concluded that both the plasminogen activator and plasmin hydrolyze lysine esters, whereas plasmin hydrolyzes arginine esters as well.

It is the purpose of this paper to present data which indicate that the proactivator described by Müllertz and Lassen may be human plasminogen. This conclusion is based on evidence obtained from a comparison of the solubility, stability and purification characteristics of proactivator and human plasminogen. Further evidence supporting this conclusion is provided through studies of the autocatalytic conversion of human plasminogen to human plasmin.

Preparations. Human plasminogen. Fraction[†] III, Human Plasma (Baxter Laboratories) and placental blood Fraction III_2 were used. Fraction III and placental blood Fraction III_2 were purified according to Kline (7), Christensen(8) or by methods described in the text. *Bovine Plasminogen.* Fifty-two liters of fresh citrated bovine plasma were warmed to 25° and with continuous stirring, 520 ml of 20% calcium chloride were added slowly. The fibrin which formed was removed by filtration through cheese cloth and 500 ml of 1% sodium ethyl mercurithiosalicylate were added to the serum filtrate. The serum was adjusted to pH 5.2, added to 10 volumes of cold distilled water and the precipitate allowed to settle at 5° for 24 hours. Most of the supernatant fluid was then siphoned off and the precipitate collected by Sharples centrifugation. Twenty grams of sodium chloride were added to the precipitate to aid in obtaining a homogeneous suspension. It was suspended in 4 liters of distilled water, adjusted to pH 6.4, and lyophilyzed. *Streptokinase*—Varidase® (Lederle) was dialyzed, adjusted to pH 6.4 and lyophilyzed. *Substrates*—Synthetic substrates were prepared as follows: L-lysine ethyl ester dihydrochloride (LEE) according to Werbin and Palm (9), and p-toluene sulfonyl arginine methyl ester monohydrochloride (TAME) according to Troll *et al.*(5). All compounds met the usual criteria for analytical purity. They were stored over phosphorus pentoxide. *Buffers*—pH 6.5 and pH 7.15 – 0.1 M phosphate buffers were used in the assays.

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* Streptokinase—abbreviated SK.

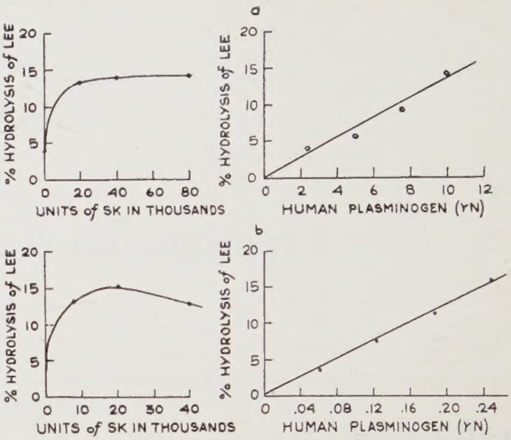

FIG. 1. (a) SK titration curve (10 γ human plasminogen) and LEE assay of a human plasminogen.[‡] The fraction was assayed using 40,000 u SK and had a calculated potency of 1.4 u/γ N. (b) SK titration curve (.25 γ human plasminogen) and activator assay of a human plasminogen.[‡] The fraction was assayed using 20,000 u SK and had a calculated potency of 60 u/γ N.

Methods. Lysine ethyl esterase assay. This assay provided a measure of the total activator and plasmin activity of SK activated plasminogen, since both enzymes hydrolyze LEE(6). When this assay is carried out in the absence of SK, it provides a measure of plasmin only. SK and varying dilutions of human plasminogen are prepared in pH 6.5 buffer. LEE is dissolved in pH 7.15 buffer at a concentration of 0.168 M immediately before use to minimize spontaneous hydrolysis(10). SK, plasminogen dilutions and buffer are warmed to 37° and then added to a series of conical centrifuge tubes in the following order: 0.5 ml pH 6.5 buffer, 0.5 ml of varying plasminogen concentrations, 0.5 ml LEE and 0.5 ml SK. Enzyme blanks for each reaction mixture are included by substitution of pH 6.5 buffer for LEE. A substrate control is composed of 0.5 ml LEE and 1.5 ml pH 6.5 buffer. The pH of all mixtures is 6.4-6.5. The reaction is started by addition of SK and after one hour at 37°, the mixtures are analyzed by the copper method(10). Values of enzyme blanks and substrate control are subtracted from values of the reaction mixtures and the difference is converted to % hydrolysis of LEE. A plot of plasminogen concentration versus % hydrolysis yields a straight line to at least 30% hydrolysis. Since the extent of hydrolysis is dependent upon the SK concentration employed for activation (Fig. 1a), the optimal SK concentration is determined by titration. The optimal SK concentration is used for assay. A typical assay plot is shown in Fig. 1a. One unit of LEE esterase is defined as that quantity of SK activated plasminogen which effects a 1% hydrolysis of LEE under the described conditions. *Activator assay.* Although an assay has been described(11), the following

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[†] Obtained from Dr. T. D. Gerlough of E. R. Squibb and Sons through the courtesy of Dr. J. N. Ashworth of American Red Cross.

TABLE I. Comparison of Human Plasma and Purified Plasminogen for Activator, TAME Esterase and LEE Esterase.

	Units/γ N			Ratio of activities		
Enzyme source	LEE esterase	TAME esterase	Activator	LEE esterase	TAME esterase	Activator
Fresh human plasma* (soluble)	.07	.08	2.5	1	1.1	35
Kline plasminogen(6); further purified (soluble)	1.4	1.2	60	1	.9	43

* Obtained through the courtesy of Mr. Frank Clarke of these laboratories.

procedure provides a more quantitative measurement of the activator of bovine plasminogen. This assay reflects the "proactivator" content of human plasminogen. Assays are carried out as described under LEE esterase assay except that bovine plasminogen, prepared in pH 6.5 buffer at a concentration of 4% is added in place of the 0.5 ml of pH 6.5 buffer. Since the incorporation of bovine plasminogen in the test system permits the use of lower concentrations of human plasminogen, hydrolysis of LEE resulting from plasminogen activator or human plasmin is negligible(11). The optimal SK concentration for maximal activation is determined for each human plasminogen fraction (Fig. 1b). It should be pointed out that in the system used for the assay of activator, there is approximately 10 times the amount of protein that is present in the LEE esterase assay. In the establishment of the optimal SK concentration (Fig. 1b) it has not been determined whether the indicated inhibition occurring at high SK concentrations is real or artifact. A plot of plasminogen versus % hydrolysis (Fig. 1b) is linear when the extent of hydrolysis does not exceed approximately 15%. A unit of activator is that quantity of SK activated human plasminogen, which results in 1% hydrolysis of LEE, under the conditions of the assay. *Tosyl arginine methyl esterase assay.* The method described by Troll *et al.*(5), employing formaldehyde in the titration of tosyl arginine was used to determine plasmin activity. However, a pH meter was employed for measuring the endpoint of the titration. One unit of plasmin is defined as that quantity of plasmin which results in 1% hydrolysis of TAME after 30 minutes, under the conditions of the assay.

Results. Comparison of human plasma and purified human plasminogen by activator, LEE esterase and TAME esterase assay. Preliminary purification experiments(7) were carried out using plasminogen of placental and blood origin, in an attempt to separate proactivator from plasminogen. It became apparent that an increase in purity of proactivator resulted in a corresponding increase in purity of plasminogen. Accordingly, plasminogen prepared by Kline's procedure(7) was further purified‡ to yield a fraction soluble between pH 2-11. This fraction was compared with a sample of fresh human plasma for activator, LEE and TAME esterase activity (Table I). The ratio of LEE esterase:TAME esterase:activator was almost identical for both fractions.

Comparison of LEE esterase and activator activity during autocatalytic conversion of plasminogen to plasmin. A fraction of human plasminogen‡ was dissolved in pH 7.6—0.1 M phosphate buffer at a concentration of 0.71 mg N/ml, preserved with merthiolate, mixed with an equal volume of glycerine§ and placed in a 37° water bath. During a 2 week incubation period, aliquots were assayed by activator and LEE esterase assay. In addition, aliquots were tested by LEE esterase and activator assay with SK omitted, so that autocatalytic plasmin and the effect of this plasmin on bovine plasminogen might be determined.

When assayed with SK, the total LEE esterase activity (plasmin + activator) was relatively constant, while proactivator, as reflected by activator assay, diminished rapidly. In contrast to the decrease in proactivator, autocatalytic plasmin (LEE esterase assay—without SK) activity increased, sug-

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‡ Unpublished data.

§ Personal communication from Dr. S. J. Sherry.

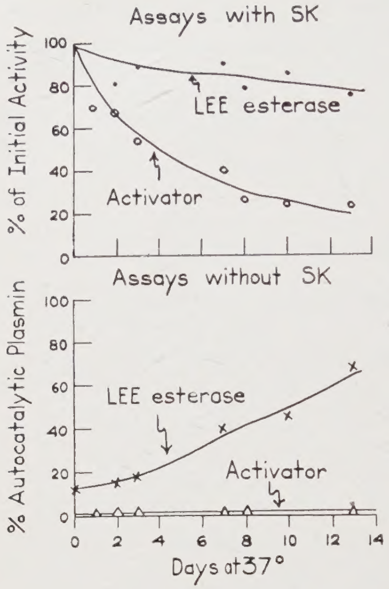

FIG. 2. Comparison of LEE esterase and activator activity during the autocatalytic conversion of plasminogen ——→ plasmin. ● Lee esterase assay (SK) represents total LEE activity (plasmin + activator); ○ Activator assay (SK) reflects proactivator content; × LEE esterase assay (without SK) represents autocatalytic plasmin only; △ Activator assay (without SK) reflects activator content in absence of SK.

gesting that either plasmin brings about the destruction of proactivator or plasmin is formed from proactivator.

Under the conditions of this experiment, plasmin did not activate bovine plasminogen (activator assay—without SK).

Stability of proactivator and plasminogen. Troll *et al.*(5,6) demonstrated a marked difference in stability of proactivator and plasminogen to heat as measured by TAME esterase and LEE esterase assays.

In view of the solubility characteristics of plasminogen, it became apparent to us that the stability of plasminogen and the instability of proactivator to heat might be related to solubility. Plasminogen preparations are generally more soluble at pH 9, the pH of the TAME esterase assay, than at pH 6.4, the pH of the LEE esterase and activator assays.

Fraction III was suspended in 0.85% sodium chloride at 6.25 mg N/ml stirred for ½ hour, adjusted to pH 7 and centrifuged. The opalescent supernatant fluid was incubated at 50° and at intervals aliquots were removed for LEE esterase, TAME esterase and activator assays. The results (Fig. 3a) show the same stability of proactivator and plasminogen by activator and LEE esterase assay; however, a greater stability of plasminogen was apparent by TAME esterase assay. This would be expected if plasminogen is more soluble in the TAME esterase assay than in the other assays.

Stability of proactivator and plasminogen in a soluble plasminogen fraction. Since the above experiment suggested a relationship between solubility and apparent stability of plasminogen, a soluble fraction was prepared. To a sulfuric acid extract(8) of Fraction III was added 3% sodium chloride. The pH was adjusted to 6.4 and the precipitate that formed was removed by centrifugation. Fifty ml of this soluble fraction, containing 0.27 mg N/ml was incubated at 50°. The solution became increasingly turbid during the incubation period. At intervals, aliquots were removed and added to an equal volume of pH 6.5 buffer. Before carrying out TAME esterase and activator assays each aliquot was filtered through a No. 2 Whatman paper to give a clear solution.

The data (Fig. 3b) show the stability of the precursor of the tosyl arginine esterase and the precursor of the activator to be identical.

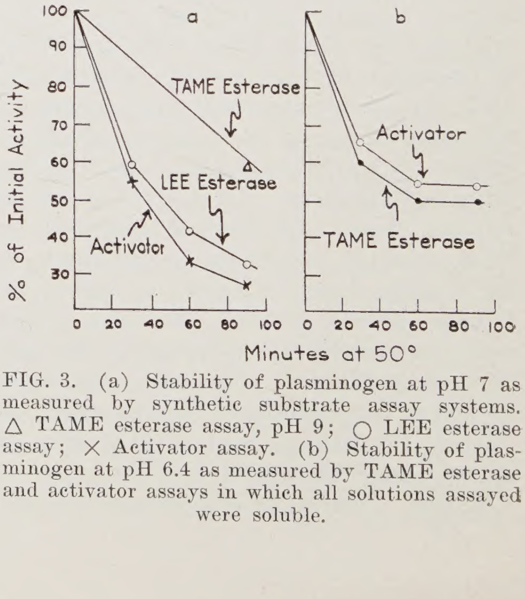

FIG. 3. (a) Stability of plasminogen at pH 7 as measured by synthetic substrate assay systems. △ TAME esterase assay, pH 9; ○ LEE esterase assay; × Activator assay. (b) Stability of plasminogen at pH 6.4 as measured by TAME esterase and activator assays in which all solutions assayed were soluble.

TABLE II. TAME and LEE Assays of Spontaneous Plasmin.*†

	TAME assays			LEE assays		
	u/ml	Mean value	% plasmin	u/ml	Mean value	% plasmin
SK	180			114		
	165	174		126	121	
	177			123		
			94			61
No SK	156			72		
	180	164		75	74	
	156			75		

* Plasmin prepared from Placental Fr III_2 by Kline's method(7).

† Prepared through the courtesy of Mr. Merle Querry of Lederle Laboratories Division, Amer. Cyanamid Co.

Evidence that activator does not hydrolyze TAME. We have confirmed the observation (6) that activator does not significantly hydrolyze TAME at pH 9. A fraction of plasminogen containing large quantities of spontaneously active plasmin (Table II) was assayed on TAME and LEE in the presence and absence of SK. TAME assays were almost identical with or without SK, but LEE assays were higher with SK. This would indicate that the activator does not significantly hydrolyze TAME at pH 9, but manifests a specificity for LEE.

Discussion. The data show that proactivator and human plasminogen have the same heat stability and purification properties. Moreover, when the plasminogen content is reduced through its autocatalytic conversion to plasmin there is also a reduction in proactivator content. These observations indicate that either proactivator and human plasminogen are closely related or they are a single substance. Purification studies are in progress in an attempt to resolve this question.

Assuming proactivator to be human plasminogen, the following mechanism for the activation of bovine plasminogen is indicated:

$$(1)\quad \text{SK} + \underset{\text{(proactivator)}}{\overset{\text{human}}{\text{plasminogen}}} \longrightarrow \underset{\text{(activator)}}{\text{SKX}}$$

$$\textit{Rapid}\ (2)\quad \underset{\text{(activator)}}{\text{SKX}} + \text{bovine plasminogen} \overset{\text{enzymatic bovine plasmin}}{\longrightarrow}$$

$$\textit{Slow}\ (3)\quad \text{Plasmin} + \text{bovine plasminogen} \overset{\text{enzymatic bovine plasmin(12)}}{\longrightarrow}$$

Evidence that plasmin is not a proactivator was provided by the finding that during the autocatalytic conversion of plasminogen to plasmin the proactivator content decreases (Fig. 2) the opposite of what would be expected if plasmin were a proactivator. It has been demonstrated(12) that, under the proper conditions, plasmin and trypsin activate bovine plasminogen; however, this activation is very slow in relation to the activation by SK plus human plasminogen. Furthermore, it is possible that the activator and plasmin might synergistically activate bovine plasminogen.

Müllertz and Lassen(4) have reported that the inability of SK to activate bovine plasminogen is due to the low proactivator content of bovine plasminogen. In view of the present evidence, the following possibilities must also be considered: (a) bovine plasminogen, because of its molecular structure, does not readily combine with SK to form activator; (b) bovine plasminogen, although molecularly the same as human plasminogen, is bound at critical centers preventing its combining with SK; (c) bovine plasminogen preparations contain inhibitors which prevent SK from combining with bovine plasminogen but do not inhibit activation by the SK human plasminogen mixture; or, (d) SK combines with bovine plasminogen to form an SKX complex which is incapable of activating bovine plasminogen or does so at a very slow rate not observable under our test conditions.

Summary. Human plasma and highly purified soluble human plasminogen contain the same proactivator:plasminogen ratio. The difference in apparent stability of plasminogen and proactivator was traced to the difference in solubility as effected by the pH of the respective assays. During the autocatalytic conversion of plasminogen to plasmin, proactivator content diminishes as plasmin is formed. A discussion of these observations is presented, wherein it is proposed that human plasminogen and the proactivator of bovine plasminogen in human plasminogen are the same factor.

The authors wish to express gratitude to Mrs.

Emma Zilko for technical assistance and to Dr. B. L. Hutchings, Dr. P. H. Bell, Dr. E. C. De Renzo and Dr. S. Gordon for helpful suggestions in preparing this manuscript.

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Sensitivity of Pertussis-Inoculated Mice to Serotonin.* (23166)

LEON S. KIND (Introduced by R. P. Walton)

Department of Microbiology, Medical College of South Carolina, Charleston.

It is well known that pertussis vaccine increases susceptibility of mice to histamine(1) and to active(2) and passive(3,4) anaphylaxis. It hardly seems likely that the concomitant sensitivity of pertussis-inoculated mice to histamine and to anaphylaxis is coincidental. However, some evidence has been presented against the importance of histamine in mouse anaphylaxis(5). Recently, Fink demonstrated that the anaphylactic contraction of isolated mouse uterus is inhibited by lysergic acid diethylamide (LSD) and reserpine(5). It was also shown that the egg-white sensitized and normal mouse uterus is 1000 times more sensitive to serotonin than to histamine. In view of the aforementioned experiments, the sensitivity of pertussis-inoculated mice to serotonin was determined.

Materials and methods. Male 20 to 27 g Tumblebrook Farm (TF) Swiss Webster mice and male 17 to 21 g Swiss Webster mice from the National Laboratory Animals Co. (NLA) were inoculated i.p. with 0.1 ml of *Hemophilus pertussis* vaccine (48.8 billion organisms/ml). Four days later (when the peak of histamine sensitivity is known to occur) pertussis-inoculated mice as well as uninoculated control animals were challenged i.p. with various doses of histamine dihydrochloride or serotonin creatinine sulfate. In the experiment involving pertussis-inoculated and uninoculated NLA mice 5 dosage groups containing 8 mice per group were employed in determining the LD_{50} of either histamine or serotonin. An exception to this procedure occurred in the uninoculated mice challenged with histamine, where only 1 group of 8 mice was challenged with a single dose of histamine. Since all animals survived, the LD_{50} was expressed as > the dose injected. In the experiment involving pertussis-inoculated and uninoculated TF mice 3 dosage groups consisting of 6 mice per group were utilized in each instance in determining the LD_{50} of each drug. Total number of animals used in both experiments was 200. Deaths were noted for a period of 24 hours. Median lethal doses were calculated by the method of Litchfield and Wilcoxon(6) and are expressed in terms of mg/kg of base.

Results. The data in Table I demonstrate that administration of pertussis vaccine greatly increases susceptibility of the mouse to the lethal effects of both serotonin and histamine. The LD_{50} and 19/20 confidence limits of serotonin were 7.9 (5.4 – 11.4) mg/kg in pertussis-inoculated TF mice and 364 (251 – 528) mg/kg in uninoculated TF ani-

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* This investigation was supported by research grant from the National Institutes of Health.

TABLE I. LD_{50} of Serotonin and Histamine in Pertussis-Inoculated and Uninoculated Mice.

Mice	LD_{50} and 19/20 confidence limits of: Histamine dihydrochloride† (mg/kg base)	Serotonin creatinine sulfate†*
Pertussis-inoculated (TF)	3.0 (2.1- 4.7)‡	7.9 (5.4- 11.4)§
Uninoculated (TF)	915 (822 -1071)‡	364 (251 -518)§
Pertussis-inoculated (NLA)	8.0 (5.0- 12.9)	12.6 (5.4- 28.4)‖
Uninoculated (NLA)	> 620	603 (568 -639)‖

* Creatinine itself (1000 mg/kg) was not lethal to pertussis-inoculated or uninoculated mice.

† Both materials were dissolved in distilled H_2O.

‡ Difference between these values is statistically significant ($p = .05$).

§ *Idem*

‖ "

mals. The LD_{50} and 19/20 confidence limits of histamine were 3.0 (2.1 – 4.7) mg/kg in pertussis-injected TF mice and 915 (822 – 1071) mg/kg in uninoculated TF controls. The LD_{50} and 19/20 confidence limits of serotonin were 12.6 (5.4 – 28.4) mg/kg in pertussis-inoculated NLA mice and 603 (568 – 639) mg/kg in uninoculated NLA animals. The LD_{50} and 19/20 confidence limits of histamine were 8.0 (5.0 – 12.9) mg/kg in pertussis-inoculated NLA mice; the LD_{50} of histamine in uninoculated NLA controls was >620 mg/kg. Since no overlapping of the confidence limits occurred, the difference between the LD_{50} doses of these drugs in pertussis-inoculated and uninoculated mice can be considered significant at the $p = .05$ level.

Discussion. It should be pointed out that the pertussis-inoculated mouse is not a generally weakened animal more susceptible to all sorts of stressor agents(3). Its altered reactivity is apparently selective. While it is more sensitive than normal mice to lethal effects of histamine(1) serotonin, anaphylaxis(3,4) Gram negative vaccines(3) and endotoxins(7,8) it is no more susceptible than normal animals to lethal effects of carbaminoylcholine(3), methacholine, epinephrine and heat stress(9). It is quite probable that both histamine and serotonin are of importance in mouse anaphylaxis. It has recently been demonstrated that edema produced in the rat by ovomucoid and other agents which damage mast cells is mediated by a combination of serotonin and histamine or closely related substances(10).

Summary. Pertussis vaccine sensitizes mice to the lethal effects of serotonin. The LD_{50} of serotonin in pertussis-inoculated NLA mice was 12.6 mg/kg as compared with 602 mg/kg in uninoculated NLA controls. The LD_{50} of serotonin in pertussis-inoculated TF mice was 7.9 mg/kg as compared with 364 mg/kg in uninoculated TF animals.

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Properdin System and Nonspecific Inhibitor of Hyaluronidase.* (23167)

Z. Hadidian, I. R. Mahler, and Marguerite M. Murphy
Departments of Pharmacology and Dental Research, Tufts University School of Medicine and Dental Medicine, Boston, Mass.

There are a number of striking similarities between the nonspecific inhibitor of hyaluronidase in normal serum (NSI)(1) and some components of the properdin system(2). NSI is heat labile, protease sensitive and can be precipitated quantitatively from a solution of low ionic strength at pH 5.2 to 5.5(3). Its action on hyaluronidase requires the presence of magnesium or cobalt ion(4) and is inhibited in solutions of high ionic strength(1). The biological significance of NSI is unknown. It is ineffective against many bacterial enzymes(5) and against those that it inhibits is not as effective *in vivo* as the specific inhibitors in serum(1). On the basis of its known properties NSI could possibly be identified with properdin or C′1. The work to be described was undertaken to test these possibilities.

Materials and methods. The methods for determination of hyaluronidase and NSI activity and the units (U) for their measurement have been described(1). Complement activity was measured by adding 0.5 ml of 1.25% sensitized sheep cell suspension to 0.5 ml saline containing the varying amounts of serum to be tested, and determining the extent of hemolysis at the end of 30 min. Zymosan (Nutritional Biochemicals) was pretreated as described(6). Human, guinea pig or rabbit serum was added to an equal volume of zymosan suspension containing .05M sodium chloride and .002M magnesium chloride and incubated for 90 min. at 37°C. The NSI and complement activity of this serum was compared with the activity of serum incubated under identical conditions without zymosan. Blood samples were taken from rabbits before and after intravenous injection of testicular hyaluronidase(7) and tested for NSI and complement activity. Blood samples obtained from guinea pigs before and 72 hr after the injection of bacterial lipopolysaccharides were tested for NSI activity. The lipopolysaccharides were from the following sources: *Salmonella typhosa, Serratia marcescens* and *Escherichia coli.*†

Results. Zymosan experiments. When serum is incubated with zymosan under proper conditions, properdin, C′3 and possible other components of complement are removed(2). In 12 experiments at pH ranging from 6.9 to 7.8 and zymosan concentrations varying from 2 to 8 mg per ml of serum there was no measurable loss in the NSI activity due to zymosan. In every instance there was a loss of complement activity. Four of these experiments are presented in Table I.

Hyaluronidase injection. Under proper conditions NSI can be removed from rabbit serum *in vivo* by intravenous injection of testicular hyaluronidase(7). Sera of such animals taken 30 min after the injection show no loss of complement activity as compared with samples taken before the injection; the NSI activity has usually disappeared completely (Table II). Furthermore, serum used in hemolysis of sensitized sheep cells does not lose any of its NSI activity.

Lipopolysaccharide injection. Eighteen guinea pigs were injected with bacterial lipopolysaccharides. Each of the 13 animals which survived showed a significant increase in the NSI titre of its serum 72 hr after injection. The increases ranged from 12% to 100% over the control values, with an average increase of about 55% (Table III). Ten days after the injection the titres were still elevated. These increases are highly significant, since in laboratory animals we have found no significant change in the serum titre of NSI from day to day. The animals that died were injected with lipopolysaccharides of

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† We are indebted to Mr. H. W. Schoenlein of Difco Laboratories for the supply of lipopolysaccharides.

TABLE I. Effect of Incubation with Zymosan on NSI and Complement Activity of Serum. Incubation was for 90 min. at 37°C.

			NSI activity, units/ml serum		Complement activity			
					ml serum producing 100% hemolysis		ml serum producing no hemolysis	
Source of serum	pH	Zymosan, mg/ml serum	Without zymosan	With zymosan	Without zymosan	With zymosan	Without zymosan	With zymosan
Rabbit	7	2	120*	120*	.05		<.025	.05
Human	7	2	27	29	.05		"	.1
"	6.9	8	27	29	.05		"	.1
Guinea pig	7.2	2	106	106	.025	.05		.025

* This activity was measured against the hyaluronidase of *Streptococcus mitis* #19; all others against testicular enzyme.

S. typhosa and *S. marcescens*.

Discussion. The properties of NSI enumerated in the introduction led us to consider the possibility that it may be identified with properdin or C′1. The experiments with zymosan show that it cannot be identified with properdin and the experiments with hyaluronidase-injected rabbits make it seem highly improbable that it can be identified with C′1. On the other hand the increase in the titre of NSI following lipopolysaccharide injection provides an additional point of similarity between NSI and properdin. All the components of the properdin system are not known, and it is probable that NSI may yet prove to be a component of this system.

Summary. The non-specific hyaluronidase inhibitor of serum (NSI) is not inactivated when serum is incubated with zymosan for 90 min at 37°C. Serum depleted of NSI does not lose complement activity. Thus it is unlikely that NSI is one of the known components of the properdin system. However, injection of lipopolysaccharides which increase the serum titre of properdin increases the titre of NSI.

TABLE II. Effect of Depletion of NSI on Complement Activity of Serum. Rabbits were given 500 to 1000 U/kg testicular hyaluronidase intrav.; serum samples were taken before and 30 min. after injection.

NSI activity, units/ml serum		Complement activity			
		ml serum producing:			
		100% hemolysis		No hemolysis	
Before inj.	After inj.	Before inj.	After inj.	Before inj.	After inj.
42	0	.2	>.2	<.05	.05
40	0	.2	.2	.05	.05
42	0	.1	.1	.03	.03
37	0	.1	.1	.04	.04
100	8	.4	.5	<.02	<.04
44	0*	.1	.1	.04	.04

* This serum contained 2.5 U/ml hyaluronidase.

TABLE III. Effect of Lipopolysaccharide Injection on NSI Activity of Guinea Pig Serum. Injections were given intraper. to animals weighing about 300 g. Serum samples were taken before and 72 hr after injection.

Lipopolysaccharide		NSI activity, units/ml	
Source	Amt, mg	Before inj.	After inj.
E. coli	1.0	111	125
	1.0	90	160
	.8	78	120
	.8	80	122
	.8	33	66
	.8	50	91
	.8	74	102
S. typhosa	1.0	46	92
	.4	74	120
S. marcescens	1.0	100	150
	.4	42	54
	.4	51	69
	.4	50	76

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Effect of Dietary Factors on Incidence of "Spontaneous" and Induced Convulsions in C_3H Male Mice.* (23168)

Y. Chiung Puh Lee, Oleg Jardetzky, Joseph T. King, and Maurice B. Visscher

Department of Physiology, University of Minnesota, Minneapolis.

This report concerns the results of observations made in connection with life span studies in mice as affected by various modifications of a basal diet. The data on convulsive seizures are being described separately because it is believed that the results may have special interest to other investigators of convulsion mechanisms in rodents. The results relating to the incidence of other specific diseases upon length of life will be reported elsewhere.

It was observed in studying singly housed C_3H male mice that convulsive seizures occur with significant frequency from 6 months of age, without intentional stimulation. The seizures are more frequent at the time of/or after handling for physical checking, weighing and cage changing. The seizures may be fatal. The frequency of seizure or the incidence of convulsive death is related to the diet used. Caloric restriction or vit. B_{12} supplementation was effective in preventing or delaying convulsive deaths while a common hydrogenated vegetable fat diet was associated with a high incidence of fatal convulsions.

Methods. Exp. 1. Two hundred and twenty C_3H male mice were selected and separated into 11 groups matched as evenly as possible for age and weight at weaning. Each group contained 20 mice which were maintained under conditions described elsewhere (1). All animals were observed until natural death or sacrifice in terminal illness. *Exp. 2.* To test effects of certain dietary factors upon induced and/or "spontaneous" convulsions, 120 weanling C_3H male mice were distributed into 6 groups. Three groups were placed on lard-containing diets: lard (17%), lard plus extra vit. E (30 mg % alpha tocopherol acetate) and low lard (5%). The other 3 groups were fed the corresponding hydrogenated vegetable fat (HVF)-containing diet: HVF (17%), HVF plus extra vit. E (30 mg % alpha tocopherol acetate) and low HVF (5%). The animals were treated as in Exp. 1, except they were housed 2 per cage. For auditory stimulation the procedure described by Hall (2) was adopted with slight modification. The stimulus was applied bi-weekly from the 15th week to the 30th week on experiment. For the administration of electric shock the method of Gellhorn and Ballin(3) was employed, modified by giving the shocks (60 cycle/sec) at regularly spaced intervals. Survivors were sacrificed at 370 to 390 days of age.

Results. The results of Exp. 1, with C_3H male mice tested on 11 different diets, are presented in Table I. It may be noted that restriction in calories alone (with normal intake of protein, minerals and vitamins) was associated with significant reduction in frequency of convulsive seizures and the incidence of convulsive deaths. The group of mice restricted both in calories and vit. E was similarly affected. The youngest animal to show a convulsion was in the group on a diet containing HVF instead of lard, the mean age at first convulsion noted was 204 days in contrast to 279 days for the control. Ninety per cent of mice fed HVF died from convulsions as compared to 55% of those on lard. Statistically the difference from control is significant at the 5% level. The average age at death in the group on the HVF diet was significantly lower than in the control group ($p < .01$), being 302 days for the former and 382 days for the latter. The earliest death in the group on HVF was at 6 months while no death was noted before 8 months in any other group. The percentage of animals showing convulsions as well as incidence of convulsive deaths decreased and the age at first seizure was increased by a supplement of vit. B_{12} in the diet, the mean age

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TABLE I. Some Dietary Effects on Incidence of Convulsive Seizure and Convulsive Death.

Diets	% of mice showing convulsions	Avg age at 1st convulsion noted (days)	% of mice dying from convulsions	Avg age at convulsive death (days)
Basal*	75	278.7 (184-457)	55	382.3 (267-503)
H-M-W salt†	80	289.5 (185-641)	60	393.9 (270-661)
Hydrogenated vegetable fat (Crisco)	95	203.8 (118-272)	90	302.2 (185-409)
Low fat (3% lard)	75	284.0 (193-427)	55	344.5 (236-439)
Amigel (replaced glucose)	65	249.2 (180-375)	40	334.0 (243-432)
Fox chow	55	335.3 (185-499)	45	456.1 (318-612)
⅓ caloric restriction	15	319.3 (237-397)	15	372.6 (304-457)
0.5% animal protein factor‡	50	387.4 (241-476)	25	399.0 (246-477)
6 γ % vit. B_{12}§	35	410.2 (260-556)	10	459.0 (432-486)
Without wheat germ oil	55	299.2 (210-442)	30	371.7 (313-442)
Idem, and ⅓ caloric restriction	5	653.0	0	

* Basal diet: Casein 28.87, glucose 34.02, lard 17.53, Brewer's yeast 8.25, alfalfa 4.12, salt 4.12 (modified Jones Foster formula), cod liver oil 2.06, wheat germ oil 1.03.

† 7% of a modified Hubbell, Mendel & Wakeman salt mixture.

‡ B_1-Con APF-3+ Pfizer.

§ General Biochemical, Inc., crystal, U.S.P.

at first convulsive seizure noted being 410 days in contrast to 279 days for the control. Only 10% of the mice had died from convulsions at a mean age of 459 days in the B_{12} group contrasted to 55% at a mean age of 382 days in the control. This difference is highly significant ($p<.01$). Furthermore, the increased age at first attack and the lowering of incidence in the group on a diet with Animal Protein Factor could be due to the presence of vit. B_{12} in that factor. As seen in Table I, the incidence of convulsive deaths in the group on Fox Chow was also high though the age at death was delayed about 2 months as compared with the control.

It should be pointed out that the difference in number of mice showing seizures or dying from convulsion between groups on a diet without wheat germ oil and the control is not statistically significant. The lower incidence of convulsive death in the group on that diet is probably due to early death of mice from a "heart failure syndrome," details of which on the diets here used have been reported(4). No difference has been noted between the groups on 2 different salt mixtures, or the group on a low fat diet.

The results of Exp. 2 designed to test susceptibility of mice on the different dietary fats by use of sound and electrical stimulation are summarized in Table II, where the number of animals found convulsing upon biweekly auditory stimulation (from 15th to 30th week on experiment) is small on all diets. However, the incidence of convulsions induced by auditory stimulation of mice fed on diets containing HVF is higher than in those fed diets containing lard. Only one out of 60 mice fed diets containing lard while 9 out of 60 mice fed diets containing HVF show audiogenic convulsions. This difference between the two groups may be significant ($p<.05$). The data do not permit a definite conclusion as to whether the early response to auditory stimulation is related to "spontaneous" seizures at a later age. Six out of 10 mice responding to auditory stimulation show "spontaneous" convulsions later. The remaining 4 mice died at an age much earlier than that at which any of the "spontaneous" convulsions appeared. On the other hand, there were 5 mice in the groups on lard diets and 11 in the groups on HVF diets showing "spontaneous" convulsions, without having responded to auditory stimulation at an earlier age. As shown in Table II, when audiogenic and "spontaneous" convulsions are considered together, the difference between the lard-fed and HVF-fed groups appears less likely to be due to chance ($p<.01$).

The results obtained from the measurement of the electroshock convulsion threshold at the age of 10 months again indicated a lower threshold in the HVF-fed groups than in the lard-fed groups. The convulsive seizures were produced by the electroshock in the

TABLE II. Mice Manifesting "Spontaneous" and/or Audiogenic Convulsions.

Type	Diets (20 mice/group)						Total No. showing convulsions in 3 groups on:	
	Lard (17%)	HVF (17%)	Lard + extra vit. E[*]	HVF + extra vit. E[*]	Low lard (5%)	Low HVF (5%)	Lard diets	HVF diets
Audiogenic	1	3	0	4	0	2	1	9
"Spontaneous"	1	5	3	4	2	7	6	16
Both or either type of seizure[†]	1	6	3	6	2	8	6	20

[*] 30 mg % alpha-tocopherol acetate.

[†] In some instances the same mouse displayed "spontaneous" and audiogenic convulsions, therefore numbers in horizontal column are not the sums of the 2 lines of numbers above them.

majority of mice on HVF-diets with 20 to 30 cycles (4/12 - 6/12 sec) while 25 to 35 cycles (5/12 - 7/12 sec) were necessary for the groups on lard-diets. The difference of the mean electroshock thresholds required to induce convulsive seizures from the groups on HVF-diets (24.2 cycles) and the groups on lard-diets (28.5 cycles) is statistically significant ($p < .01$). The mortality in the former is slightly higher (37%) than in the latter (22%). All the results suggest that the mice on a HVF-diet are more susceptible to intentional stimulation of the types used.

Discussion. These results indicate that the dietary regimen exerts an effect on the "spontaneous" convulsions in C_3H male mice, although environmental conditions, such as caging, handling and others are determining or precipitating factors(1). There is no explanation as to why the incidence of convulsions is higher on a diet containing hydrogenated vegetable fat. Davenport and Davenport(5) described a reduction of electroshock threshold in normal rats fed a diet supplemented with extra fat (Crisco). In the studies herein reported a quantitative difference in fat intake did not influence the frequency of "spontaneous" convulsive seizures. However, when animals were fed a diet containing HVF instead of lard their electroshock threshold was reduced.

The mechanism of caloric restriction alone, in protecting against convulsions remains unsolved. However, increased resistance to a number of diseases or stress has been noted in underfed or caloric restricted rats or mice(4, 6,7). Geyelin(8) reported that fasting reduced the incidence of seizures in epileptic patients, and the effect has been ascribed to the resulting ketosis. The Davenports(9), on the other hand, found a lowering of electroshock threshold by dietary restriction in pyridoxin-deficient rats.

The low frequency of fatal convulsions or convulsive seizures in the group on a diet supplemented with extra vit. B_{12} indicated further the importance of nutritional factors in relation to the convulsive attacks.

The basal diet contained 25% crude casein. During the course of this life-span experiment many different batches of casein were used, none of which was assayed for B_{12} content. Lewis, *et al.*(10) found that the B_{12} content of crude casein was 3 μg/100 g. Excluding special materials such as liver, rumen contents, etc., this value for crude casein compares well with other natural products such as meats, egg yolk and cheese reported by these investigators. Collins, *et al.*(11) found little difference in the B_{12} content of milk from different pure breeds of cows. The values found for commercial cow's milk, raw and pasteurized, were somewhat lower than for the pure breeds studied (raw 3.4; pasteurized 4.0; pure breeds 6.6 μg/l).

All groups of mice fed the basal diet *ad libitum* were heavier after 9 weeks of age than the group fed commercial fox chow. This does not suggest that the basal diet was deficient in B_{12}. It is also known[†] that the basal diet is adequate for normal lactation in the A mouse. Nevertheless, the possibility remains that the B_{12} content of unsupplemented basal diet was sub-optimal for the C_3H mouse. In addition to the observed decreased incidence of convulsions on B_{12} supplement, this group showed the highest body weight of any group (after 15 weeks of age). Whether or not the unsupplemented basal diet contained the optimal amount of B_{12} for our C_3H mice cannot be determined from the data available to us.

[†] Unpublished data.

As seen in the Tables the incidence of "spontaneous" convulsive seizures in the first group of experimentals is higher than that obtained from the Exp. 2 regardless of diet. This difference may be attributed to the differences in caging. The first experimental group of mice was caged singly while the latter group was kept two per cage. It has been reported(1) that when C_3H male mice are housed 2 per cage, the incidence of "spontaneous" seizures was reduced and with twenty to a large cage the incidence was negligibly small. A factor of a "social" nature appears to be implicated.

Summary. 1. The incidence of "spontaneous" convulsions and of convulsive deaths in C_3H male mice is greatly reduced by caloric restriction or by supplement of vit. B_{12} in the diet fed *ad libitum.* Substitution of hydrogenated vegetable fat for lard decreased the age of onset of convulsions and increased the incidence of convulsive deaths. 2. Studies with auditory and electric stimulation appear to confirm the existence of a difference between the effect of HVF and of lard.

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Influence of Thrombin on Rate of Prothrombin Conversion. (23169)

DONALD G. THERRIAULT, JOHN L. GRAY, AND H. JENSEN
Biochemistry Department, U. S. Army Medical Research Laboratory, Fort Knox, Ky.

There appears to be general agreement among investigators of blood clotting that thrombin accelerates its own formation from prothrombin. However, the specific mode of this action of thrombin is, at present, not completely resolved. The present work is concerned with an attempt to show that traces of thrombin will accelerate the formation of a prothrombin-conversion factor. Inasmuch as it has been found that plasma accelerator-globulin*(1,2) and probably also other clotting factors(2) may be associated with platelets, a phospholipid preparation from beef brain corresponding to the phosphatidyl ethanolamine fraction of Folch(3) has been used as a substitute for platelets.

Materials. Antihemophilic Factor (AHF) and Plasma Accelerator-Globulin (PAcG): The method used for preparation of these 2 factors was a combination of the procedures described by Bidwell(4) and by Lewis and Ware(5). $BaSO_4$-treated oxalated bovine plasma was diluted 1:20 with distilled water and the pH adjusted to 5.5. Precipitate from 200 ml plasma was dissolved in 90 ml of 0.9% NaCl (pH 7.0) and the solution adjusted to a concentration of 10% sodium citrate by the dropwise addition of a 40% sodium citrate solution. Precipitate and supernatant solution were then worked up separately. Antihemophilic factor (AHF); precipitate was taken up in 75 ml of 0.9% NaCl (pH 7.0), heated for 10 min at 52°C and precipitate discarded. Supernatant fluid was taken to 0.3 saturation of $(NH_4)_2SO_4$ with solid $(NH_4)_2SO_4$. Precipitate was dissolved in a small amount of 0.9% NaCl (pH 7.0) and dialyzed in the cold against 0.9% NaCl (pH 7.0). The solution was then lyophilized. Plasma accelerator-globulin (PAcG); supernatant solution (described above) was brought to 25% sodium citrate concentration. Precipitate was dissolved in 70 ml of cold 0.9% NaCl (pH 7.0) and precipitated between 0.33 to 0.5 saturation of $(NH_4)_2SO_4$ by the addition of a saturated $(NH_4)_2SO_4$ solution. Precipitate was dissolved in a small amount of 0.9% NaCl (pH 7.0) and dialyzed in the cold against 0.9% NaCl (pH 7.0). This material was frozen in small aliquots and used as a stock solution. *Prothrombin*: Prepared according to the procedure described by Surgenor *et al.*(6). The prothrombin preparation, assayed according to the method of Ware and Seegers(7), was found to contain 100 units per mg of protein. *Serum eluate preparation* (*SE*): Previously frozen rabbit serum was thawed, allowed to stand at room temperature for 24 hours and 0.1 M sodium oxalate added in the ratio of 1 volume to 9 volumes of serum. After addition of $BaSO_4$ (100 mg/ml) the mixture was stirred for 20 min. The $BaSO_4$ was collected by centrifugation and washed with an equal volume of 0.9% sodium chloride-sodium oxalate mixture (9 parts of 0.9% NaCl to 1 part of 0.1 M sodium oxalate) and then with 0.9% NaCl. The $BaSO_4$ was eluted twice with 0.1 M sodium citrate equal to 1/10 the original volume of serum. The eluates were combined, brought to 50% saturation with solid ammonium sulfate and kept at 4°C overnight. The precipitate was filtered off, collected in a small amount of distilled water and dialyzed in the cold against distilled water until completely free of ammonium sulfate. After centrifugation the precipitate was extracted with 0.1% ammonium carbonate, centrifuged and the supernatant solution lyophilized. The proconvertin activity of this preparation, determined according to the method of Owren and Aas(8), was found to be 400 units per mg. One unit of proconvertin was arbitrarily defined as that amount of preparation which will give a clotting time equal to that of 0.1 ml of a 1:50 dilution of normal rabbit plasma as assayed by the above method. *Thrombin*: Parke, Davis Topical Thrombin (1% solution in oxalated 0.9% NaCl) was stirred with $BaSO_4$ (50 mg/ml) for 20 min at room temperature. The $BaSO_4$ was centrifuged down, the decanted supernatant solution adjusted to pH 7.2 with 2 N NaOH and taken to 38% saturation with solid ammonium sulfate. After centrifugation the supernatant solution was adjusted to pH 5.2 with 2 N HCl and taken to 70% saturation with solid ammonium sulfate. The precipitate obtained after centrifugation was dissolved in a small amount of water and dialyzed against cold distilled water until free of ammonium sulfate. The dialysate was centrifuged and the supernatant solution stored in small aliquots in the frozen state. Thrombin activity was equal to 250 u/ml with a protein concentration of 3.56 mg/ml. *Fibrinogen*: Fibrinogen was prepared according to the procedure described by Ware *et al.*(9). The stock solution was diluted 1:20 with 0.9% NaCl adjusted to pH 7.0. Final concentration of fibrinogen was 0.16%. *Phospholipid*: Folch's (3) method was used for the preparation of a cephalin fraction corresponding to phosphatidyl ethanolamine.

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* For a different terminology of factors referred to in this paper reference is made to Appendix, The Coagulation of Blood. Methods of Study. Edited by L. M. Tocantins, Grune and Stratton, N. Y., 1955.

Methods. The rate of prothrombin conversion was determined in two ways: A "1-stage" procedure in which the reactants involved in prothrombin conversion were mixed simultaneously, and a "2-stage" procedure in which certain reactants supposedly involved in the formation of a prothrombin-conversion factor were preincubated prior to addition of the prothrombin preparation. The testing procedure consisted of preparing a reaction mixture of different factors and measuring amount of thrombin formed at various time intervals of incubation at 26°C by adding a measured amount of the reaction mixture to a fibrinogen solution at 37°C. Activity of the reaction mixtures used in this investigation was determined by degree of conversion of prothrombin to thrombin. *One-stage reaction*: The reaction mixture consisted of the following: prothrombin 0.2 ml (5 mg/ml), serum-eluate 0.2 ml (0.2 mg/ml), phospholipid 0.05 ml (1 mg/ml), plasma accelerator-globulin 0.25 ml (2 mg/ml), antihemophilic factor 0.5 ml (0.4 mg/ml), thrombin 0.02 ml (5 NIH units), and 0.125 M $CaCl_2$ 0.1 ml. The final volume was adjusted to 2.70 ml by the addition of 0.9% NaCl (pH 7.0); the final reaction mixture had a pH of 7.0. At various time intervals of incubation at 26°C 0.1 ml of the reaction mixture was added to 0.4 ml of fibrinogen at 37°C. When the clotting times (as obtained by the tilt method) were below 15 sec, the 0.1 ml portions of the reaction mixture were suitably diluted with 0.9% saline (pH 7.0) before addition to the fibrinogen solution. The recorded clotting times were interpreted in terms of National Institutes of Health (NIH) Thrombin units from a curve prepared with a Standard NIH Thrombin preparation.[†] *Two-stage reaction*: This reaction was carried out with the same preparations in identical proportions as described for the 1-stage reaction. However a preliminary reaction was allowed to take place by first incubating serum-eluate, phospholipid, plasma accelerator-globulin, antihemophilic factor, and $CaCl_2$ in the absence or presence of thrombin for 10 min at 26°C before adding the prothrombin. After addition of prothrombin the generation of thrombin at 26°C was followed in the same manner as described above for the 1-stage reaction.

Results. The results reported here are average values of at least 8 separate experiments. Variation in any given series of experiments never exceeded 10%. Though the results reported are for a period of only 15 minutes, experimental measurements were recorded for a period of 60 minutes. It was found that the percent conversion reached a maximum within 15 minutes and remained constant throughout the 60 minute period. A reaction mixture containing serum-eluate, phospholipid, $CaCl_2$ and prothrombin yielded only traces of thrombin. However, if PAcG is added to the above reaction mixture (Fig. 1 curve B) an average of 71% of the prothrombin present is converted to thrombin. If in place of PAcG, AHF is added to the reaction mixture (Fig. 1 curve C), again only about 77% of the prothrombin is converted to thrombin. It is not until AHF and PAcG are present together in the reaction mixture containing serum-eluate, phospholipid, $CaCl_2$ and prothrombin (Fig. 1 curve A), that complete conversion of prothrombin to thrombin takes place.

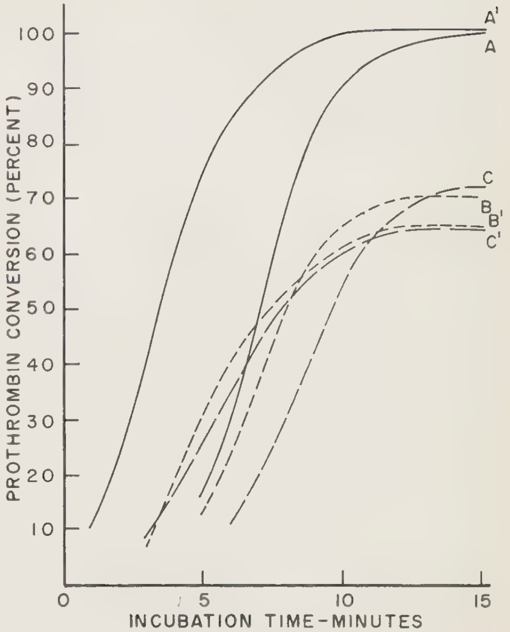

FIG. 1. Influence of thrombin on rate of prothrombin conversion (one-stage). A = Complete reaction mixture without thrombin. A' = Complete reaction mixture with thrombin. B = Reaction mixture minus AHF without thrombin. B' = Reaction mixture minus AHF with thrombin. C = Reaction mixture minus PAcG without thrombin. C' = Reaction mixture minus PAcG with thrombin.

It will be noticed in Fig. 1 that in each of the reactions there is a definite lag period during which there is no detectable conversion of prothrombin to thrombin. However, after a definite period of time has elapsed and thrombin formation has started, conversion of prothrombin takes place quite rapidly. It can also be seen (Fig. 1) that addition of thrombin (5 NIH units) to either one of the reaction mixtures decreases this lag period. In the complete reaction mixture, the lag period is almost entirely eliminated. It was found that addition of thrombin did not reduce the lag period or enhance conversion of prothrombin in the reaction mixture containing only serum-eluate, phospholipid, $CaCl_2$ and prothrombin. It would seem, therefore, that thrombin affects the activity of PAcG and AHF. Fig. 1 also shows that addition of thrombin caused a faster conversion of prothrombin to thrombin but did not increase amount of thrombin formed.

[†] Kindly supplied by Biologics Control Laboratory, N.I.H., Bethesda, Md.

Since the activities of the PAcG and AHF preparations seemed to be alike in this test reaction, and since the effect of thrombin on each of these preparations appeared to be similar, varying concentrations of both factors were used in the reaction mixture. If both preparations were one and the same factor, doubling the concentration of one factor in the absence of the other should have increased degree of prothrombin conversion. It was found that degree as well as rate of conversion of prothrombin to thrombin remained the same when the amount of AHF was doubled. Similarly, twice the amount of PAcG in a reaction mixture containing no AHF did not increase extent or rate of conversion of prothrombin. This would indicate that AHF and PAcG preparations employed in these studies are different agents and that both factors must be present in the reaction mixture to attain complete conversion of prothrombin to thrombin.

If rate of prothrombin conversion obtained in a 1-stage procedure using a complete reaction mixture minus thrombin is compared with rate of prothrombin conversion in a 2-stage procedure using the same agents (Fig. 2 curves A and B), it may be seen that preincubation of serum-eluate, AHF, PAcG, phospholipid and $CaCl_2$ shortened the lag phase. These results seem to indicate that incubation of phospholipid, AHF, PAcG, serum-eluate and calcium ions yields a prothrombin-conversion factor. In the absence of phospholipid or of serum-eluate there was found to be no formation of a prothrombin-conversion factor. The activity of the serum-eluate preparation may be due to the presence of plasma thromboplastin component* or of proconvertin* or of both.

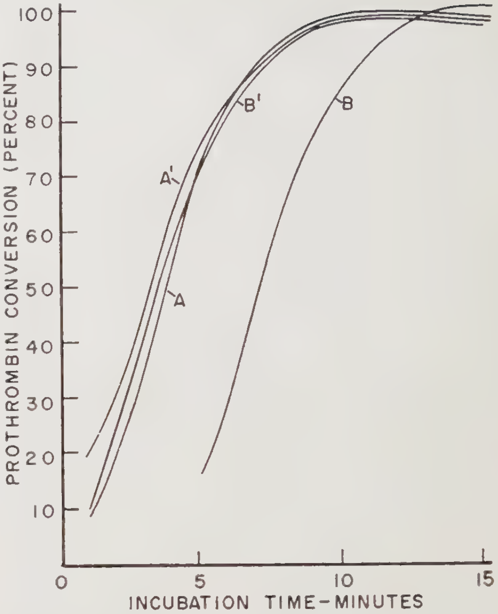

FIG. 2. Influence of thrombin on the formation of prothrombin conversion factor (two-stage). A = 2-stage, complete reaction mixture without thrombin. A′ = 2-stage, complete reaction mixture with thrombin. B = 1-stage, complete reaction mixture without thrombin. B′ = 1-stage, complete reaction mixture with thrombin.

It is interesting to note that the lag period (Fig. 2) was greatly shortened in the 1-stage reaction containing all the reactants including thrombin and in the 2-stage reaction containing a complete reaction mixture with or without thrombin. It might seem from inspection of the curves A and A′ of Fig. 2 that the 2-stage reaction mixture in the presence of thrombin produced the prothrombin-conversion factor at a faster rate than did the 2-stage reaction mixture without thrombin. However, the difference was too small to allow any definite conclusions.

Discussion. The results on rate of prothrombin conversion in a 1-stage procedure (Fig. 1) seem to indicate that action of PAcG as well as that of AHF is enhanced by traces of thrombin. These observations support the finding of Seegers and his associates(10) demonstrating the importance of traces of thrombin on rate and completeness of prothrombin conversion under various experimental conditions in a 1-stage reaction procedure. They expressed the opinion that thrombin converted PAcG in the reaction mixture to the more active serum accelerator-globulin. According to Lanchantin and Ware(11) thrombin has a specific proteolytic effect on plasma accelerator-globulin which is associated with a significant increase in accelerator-globulin activity. The above results are also in agreement with the assumption of Quick and his associates(12) that thrombin activates thromboplastinogen (AHF). As can be seen from Fig. 1, traces of thrombin shortened the lag period of prothrombin conversion. It appears that once a certain amount of thrombin (either added as such or formed during the reaction) is present, the conversion of prothrombin to thrombin proceeds at a comparatively rapid rate.

From the data presented here, it appears that thrombin will accelerate formation of the prothrombin-conversion factor. Fig. 1 and 2 illustrate that the slopes of the curves are the same in the absence or presence of thrombin. This indicates that rate of prothrombin conversion is the same in all cases. The effect of thrombin was to shorten the lag period of the start of thrombin formation. This lag period seems to be a function of rate of formation of the prothrombin-conversion factor. In the presence of thrombin the rate of formation of the prothrombin-conversion factor becomes accelerated. Once sufficient prothrombin-conversion factor is formed, the conversion of prothrombin to thrombin takes place quite rapidly. Inasmuch as traces of thrombin will apparently accelerate formation of a prothrombin-conversion factor, a proper evaluation of a thromboplastin generation test should take into account the possible presence of trace amounts of thrombin derived from prothrombin in the serum employed.

Summary. Both antihemophilic factor and plasma accelerator-globulin are necessary to obtain complete conversion of prothrombin in the presence of a $BaSO_4$-eluate preparation from serum, phospholipid-thromboplastin and $CaCl_2$. In the presence of traces of thrombin conversion of prothrombin to thrombin becomes greatly accelerated. Results indicate that thrombin greatly accelerates formation of a prothrombin-conversion factor. This influence of thrombin appears to be mediated through its activating effect on plasma accelerator-globulin and, perhaps, also on the antihemophilic factor.

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Analgesic Properties of Xylopropamine.* (23170)

S. C. Harris and R. C. Worley

Department of Physiology and Pharmacology, Northwestern University Dental School, Chicago, Ill.

Information that sympathomimetic drugs are analgesic has been accumulating for more than 50 years. Thirteen earlier reports (beginning in 1904) of clinical and laboratory experiences were cited by Ivy, *et al.*(1), when they confirmed Weber's(2) observation that epinephrine given intracarotidly conferred profound analgesia without alteration of other conscious behavior. Ivy used dogs, Weber used cats. Epinephrine, ephedrine, and amphetamine were employed in the several studies referred to. More recently, additional evidence has accrued. Leimdorfer(3) produced profound clinical analgesia by intraspinal administration of epinephrine. Amphetamine potentiation of morphine analgesia has been reported by Goetzl and Burrill in animals(4) and man(5). That amphetamine alone can elevate the pain threshold has been reported by Burrill, Goetzl, and Ivy(6) and by Harris and Worley(7). Physiologically occurring epinephrine may participate in the production of analgesia by certain established compounds (like morphine). Friend and Harris(8) and Gross, *et al.*(9), found ablation of adrenal medullae to reduce the analgesic potency of said compounds. Exactly what "analgesia" may be remains a mystery. That the pain experience is a complex and variable combination of perception and interpretation seems to be a general observation acceptable to most algesimetricians and clinicians. This is reflected in their reports that some "analgesics" do not reduce the pain sensation but make it more tolerable to the patient or render him indifferent to the pain, *i.e.*, patient "suffering" is reduced while the perceptual experience persists. Such a mechanism of "analgesia" has been attributed frequently to the opiates and to the sympathomimetics. Evidently, indifference to pain is thought to be favored by narcosis and/or analepsis—an easily enough formulated hypothesis but one not particularly tenable. Analgesia with narcotics is enhanced by the addition of analeptic sympathomimetics(5) and the addition of barbiturates to sympathomimetic analeptic analgesics does not diminish their analgesic effectiveness(7). An amphetamine compound which is analgesic but not stimulating might further disassociate analepsis from analgesia, as well as providing certain clinical conveniences. According to reports from the manufacturers' laboratories, xylopropamine sulfate[†] (1-(3,4-Dimethylphenyl)-2-Aminopropane) is one-third to one-fifth as stimulating as dextro-amphetamine. Thus, evaluation of its analgesic properties seemed appropriate.

Method. Each of 8 male human volunteers served for 4 experiments, in which the threshold to experimentally induced pain was determined at intervals before and after oral medication according to the method of Harris and Blockus(10). *Medications* were: A. placebo, B. 5 mg xylopropamine sulfate, C. a "dry-run" in which no capsule was taken, and D. 10 mg xylopropamine sulfate. All capsules were prepared to look alike and were taken with water at least 4 hours postprandial. *Medication sequence* was according to a "latin-square" design so that each treatment was the first for 2 subjects, second for 2 subjects, etc. *Stimuli* were square waves of one millisecond duration automatically generated every 0.5 second. Intensity of the stimulus was slowly increased in one millivolt steps until the subject signaled that he felt pain. The process was then immediately repeated. Both values, so obtained, were recorded. The *stimulus sites* were metal restorations in vital teeth. Only "Class I" fillings (in the occlusal plane with no overlap beyond the crown of the tooth) were used. The same site was always used in each man's mouth. Before placing the electrode against the "filling," the surface was carefully dried with ethyl alcohol, cotton rolls being inserted on

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* Aided in part by grant from Hoffmann-La Roche.

[†] Supplied by Hoffmann-LaRoche, Inc.

TABLE I. Average Increments in Millivolts of Pain Thresholds after Placebo, "Dry-run", 5 mg and 10 mg Oral Doses of Xylopropamine.

Min. after medication	Placebo	"Dry-run"	Xylopropamine 5 mg	Xylopropamine 10 mg
30	15.6	-142.0	- 68.5	-72.5
60	-314.4	- 75.0	-192.5	109.4
90	-269.1	-292.5	-214.4	105.6
120	3.8	-367.5	- 43.1	168.1
150	-206.2	-416.2	- 11.9	565.0
180	-267.0	-395.0	.6	437.0

the labial border of the site. It is imperative that the surface of the tooth be dry to prevent dissipation of current around the pulp rather than through it. The indifferent electrode from the stimulator is placed in a beaker of saline solution with one of the subject's hands. *Intervals* were 30, 15 and 1 minutes prior to ingestion of drugs and 30, 60, 90, 120, 150, and 180 minutes after. The interval between experimental sessions was never less than 48 hours. Bias was controlled by "triple blind" discipline. Except for the "dry-run," neither operator nor subjects knew medication identities until the entire study was completed. Which subject was which was unknown to the director of the study. This control was achieved by labeling in advance 4 small envelopes for each subject with his number in a Roman numeral (Subject I) and the experiment number in an Arabic (Exp. 1). These 32 envelopes were filled according to the protocol and stapled shut. This process was checked 3 times for accuracy. To confirm proper chronology of treatment, when an envelope was opened by a subject, it was returned to the operator who stamped the date on it. To be assured against the need for emergency identification of a medicament, it is standard practice in this laboratory to provide the operator with an opaque, sealed envelope containing the code. Appropriate antidotes or other emergency materials are always immediately available.

Results. The average value of each individual dial reading in the raw data was converted to millivolts from the calibration curve of the stimulator and recorded on a master table for each medication. These data, in turn, were simplified further by recording the post-medication increments from the average of the second and third premedication thresholds, and then recorded in Table I as average increments in millivolts per treatment and interval.

Whether or not the differences evident in Table I are real cannot be determined with confidence by inspection. Accordingly, the increment data from which Table I was derived were subjected to analyses of variance.

In Table II is the summary of such an analysis on the entire data. Though there is no significant difference between the increments following dry-run, placebo and a 5 mg dose of xylopropamine sulfate, the increments following these 3 treatments are significantly inferior ($P<0.05$) to the increments following 10 mg doses of xylopropamine sulfate. It is to be pointed out that such an analysis actually compares the *average* increments throughout the entire postmedication period, even though it is quite evident that no effects really appear until more than one hour after medication.

To make the analysis more sensitive, an analysis of variance was performed on the same data by observation intervals. From the results of these analyses (Table III) it may be seen that the same conclusions as above pertain to the 150 and 180 minute postmedication intervals. A moot question thus far in algesimetry is whether to express threshold changes as absolute increments or as percent of premedication thresholds. Therefore, these data have been reevaluated

TABLE II. Summary of Analysis of Variance.

Source	Degrees freedom	Mean square	"F" ratio
Major			
Subjects	7	1053029	1.142
Treatments	3	2215786	2.403
(ABC)	(2)	(898753)	.974
(ABC)(D)	(1)	(5748606)	6.233*
Subj. × treat.	21	922266	
Minor			
Times	5	90899	
" × subj.	35	120099	
" × treats.	15	251941	
S × T × T	105	221390	

* $P <.05$.

TABLE III. Summary of Analyses of Variance by Postmedication Intervals.

Interval (min.)	Source	Degrees freedom	Mean square	"F" ratio
30	Subjects	7	84541	1.683
	Treatments	3	33244	.661
	S × T	21	50221	
60	Subjects	7	322723	.829
	Treatments	3	260431	.670
	(ABC)	(2)	(229227)	.580
	(ABC)(D)	(1)	(552066)	1.419
	S × T	21	389040	
90	Subjects	7	464094	1.607
	Treatments	3	273992	.949
	(ABC)	(2)	(25726)	.089
	(ABC)(D)	(1)	(796251)	2.757
	S × T	21	288768	
120	Subjects	7	258511	1.801
	Treatments	3	402522	2.804
	(ABC)	(2)	(326991)	2.278
	(ABC)(D)	(1)	(553584)	3.855
	S × T	21	143566	
150	Subjects	7	382393	.595
	Treatments	3	1395032	2.107
	(ABC)	(2)	(327201)	.509
	(ABC)(D)	(1)	(3530693)	5.490*
	S × T	21	643164	
180	Subjects	7	145170	.283
	Treatments	3	1081390	2.107
	(ABC)	(2)	(325988)	.635
	(ABC)(D)	(1)	(2592194)	5.051*
	S × T	21	513157	

* P <.05.

as percentile changes.

New tables (not appended) were prepared so that each original data cell was an expression of the increment as a percent of the premedication threshold. The results of this procedure are presented in Table IV, which is of somewhat different character than Table I with respect to the effects of "dry-run," placebo and 5 mg of xylopropamine.

The same statistical procedures as above were applied to these data and appear summarized in Tables V and VI. Again, the average effect of 10 mg of xylopropamine was superior (P<0.05) to the average effects of the other 3 treatments. When broken down by intervals, this relationship first appeared at 90 min. postmedication and persisted through to the final reading.

TABLE IV. Average Increments in Percent of Pain Thresholds after Placebo, "Dry-run", 5 mg and 10 mg Oral Doses of Xylopropamine.

Min. after medication	Placebo	"Dry-run"	Xylopropamine 5 mg	Xylopropamine 10 mg
30	2.26	–1.51	– .08	.28
60	–1.34	.43	–1.31	4.47
90	– .87	–3.82	– .14	10.07
120	4.91	–6.66	6.14	9.16
150	3.69	–6.54	6.05	19.91
180	.80	–7.32	4.47	14.88

TABLE V. Summary of Analysis of Variance on % Values.

Source	Degrees freedom	Mean square	"F" ratio
Major			
Subjects	7	625.2650	1.052
Treatments	3	1591.1029	2.678
(ABC)	(2)	(642.8832)	1.082
(ABC)(D)	(1)	(3487.5422)	5.869*
(AB)	(1)	(21.5177)	.036
(C)(AB)(D)	(2)	(2375.8955)	3.998*
Subj. × treat.	21	594.2438	
Minor			
Intervals	5	142.4264	
Subj. × int.	35	109.5959	
Treats. × int.	15	158.6389	
S × T × I	105	110.9249	

* P <.05.

A different partitioning of treatment variance was suggested by Table IV. In this treatment, effects of A and B (Placebo and 5 mg xylopropamine) were compared and found to be similar at all intervals. However, there were statistically significant differences between treatments C (dry-run), A and B together and D (10 mg xylopropamine) 90 and 120 minutes after medication. At 150 and 180 minutes, the probability of coincidence causing the differences observed has risen to an estimated 0.07.

Discussion. The findings of this study strongly indicate that xylopropamine, in 10 mg oral dosage, is analgesic. The latency of response is reminiscent of experience obtained with another sympathomimetic—Daprisal(7). A possible explanation of this delay in effect, is that by its local vasoconstricting effect, the sympathomimetic delays its own absorption. Efforts to prove or disprove this hypothesis have not been made. The present evidence demonstrates a similarity of analgetic activity between xylopropamine and amphetamine. If the disparity between their

TABLE VI. Summary of Analyses of Variance by Postmedication Intervals on % Values.

Interval (min.)	Source	Degrees freedom	Mean square	"F" ratio
30	Subjects	7	20.8341	.464
	Treatments	3	19.3750	.432
	S × T	21	44.8773	
60	Subjects	7	260.1349	2.019
	Treatments	3	59.7446	.464
	S × T	21	128.8230	
90	Subjects	7	187.1359	2.144
	Treatments	3	293.1162	3.358*
	(ABC)	(2)	(30.3779)	.348
	(ABC)(D)	(1)	(818.5928)	9.379†
	(AB)	(1)	(2.1025)	.024
	(C)(AB)(D)	(2)	(438.6231)	5.026*
	S × T	21	87.2780	
120	Subjects	7	228.0122	2.389
	Treatments	3	506.7374	5.309*
	(ABC)	(2)	(398.6667)	4.176*
	(D)(ABC)	(1)	(722.8788)	7.573*
	(AB)	(1)	(6.0393)	.063
	(C)(AB)(D)	(2)	(757.0864)	7.932†
	S × T	21	95.4441	
150	Subjects	7	323.0345	.686
	Treatments	3	949.1065	2.018
	(ABC)	(2)	(358.2576)	.762
	(D)(ABC)	(1)	(2130.8041)	4.532*
	(AB)	(1)	(22.2548)	.047
	(C)(AB)(D)	(2)	(1412.5323)	3.004
	S × T	21	470.2159	
180	Subjects	7	154.0929	.506
	Treatments	3	678.6871	2.227
	(ABC)	(2)	(291.1062)	.955
	(D)(ABC)	(1)	(1453.8489)	4.771*
	(AB)	(1)	(54.1328)	.178
	(C)(AB)(D)	(2)	(990.9642)	3.252
	S × T	21	304.7322	

* $P < .05$. † $P < .01$.

analeptic effects can be extended to include human quantitative evidence, interesting new hypotheses on the mechanism of analgesia may be formulated: *e.g.,* that sympathomimetic drugs in some way diminish the intensity of pain sensations reaching the level of perception.

Summary. 1) A new sympathomimetic drug, xylopropamine sulfate (1-(3,4-Dimethylphenyl)-2-Aminopropane), has been found capable of elevating the threshold of human subjects to experimentally induced pain. The method employed was electrical stimulation of the tooth pulp. There were 8 subjects and the experiment was conducted under "double blind" discipline. Five and 10 mg oral doses of xylopropamine were compared with a placebo and a "dry-run." The average effect of the 10 mg dose was statistically superior ($P < .05$) to the effects of the other treatments from 90-180 minutes after medication. 2) It is provocative that xylopropamine is analgesic but apparently not analeptic, while amphetamine is both. It suggests that the two effects on the sensorium could be independent.

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Conversion of Porphobilinogen to Porphyrins by Normal Rat Liver Homogenate.* (23171)

SAMUEL SCHWARTZ AND C. J. WATSON

Department of Medicine, University of Minnesota Hospitals, Minneapolis.

Westall's crystallization of porphobilinogen (PBG) from urine of porphyria patients(1) has stimulated a number of studies which indicate that this compound is the basic pyrrole unit formed in the biosynthesis of porphyrins and of heme compounds. Chemical and other studies(2,3) have shown that it is a monopyrrole containing propionic and acetic acid side chains and an amino-methyl group on one alpha carbon atom. When heated in dilute acid, PBG is readily converted to uroporphyrin(1,2). When incubated with hemolyzed chicken erythrocytes, coproporphyrin and large amounts of protoporphyrin are formed, in addition to uroporphyrin as shown by Falk *et al.*(4). Similar results have been reported by Bogorad and Granick for PBG incubated with broken cell preparations of Chlorella(5).

The present studies were undertaken to test the ability of normal rat liver homogenate to convert PBG to porphyrin. This was deemed of fundamental interest because of the previously demonstrated primary role of the liver in the genesis of those forms of human(6) and experimental(7) porphyria characterized by the excretion of large amounts of PBG. Similar studies employing livers of Sedormid-treated rats are reported in the companion paper(8).

Methods. A. Incubated samples. The PBG employed was crystallized by a slight modification(9) of Westall's procedure, from urine of patients with acute intermittent porphyria. The livers of 3 to 6 normal adult male rats were perfused with saline until the returns were nearly colorless. Three ml of cooled 0.1 molar phosphate buffer pH 7.4 and sufficient penicillin and dihydrostreptomycin to yield final concentrations of 500 units and 500 μg/ml respectively, were added/gram of pooled rat liver. This material was homogenized in Waring blender for 2 minutes. Twenty-five ml portions were pipetted into separate Erlenmeyer flasks and PBG freshly dissolved in 5 ml phosphate buffer was added to each. The "0" time porphyrin content of the control flask was determined at once, or, in some instances, after keeping at −15°C for 12-48 hours. The other flasks were incubated at 37°C for varying periods, as noted below, and then kept at −15°C until analyzed. B. Analytical procedures. The homogenate was poured with constant shaking into 12 volumes of a 4:1 mixture of ethyl acetate:glacial acetic acid. The flocculant precipitate was removed on a medium sintered glass filter, and ground three times with small additional portions of the ethyl acetate:acetic acid mixture. (Subsequent extractions of the residual precipitate with a 19:1 mixture of methanol:sulfuric acid revealed that it contained only traces of porphyrin.) One-half ml of a 1% iodine solution in alcohol was added to the combined filtrate to convert Ehrlich negative (non-PBG) "precursors" to porphyrin (10). The total porphyrin was then extracted with successive small portions of 3 N hydrochloric acid until no further red fluorescence was demonstrated in the extract in ultraviolet light. The subsequent fractionation and quantitative analysis of the copro- and proto-porphyrin was similar to that described elsewhere for the erythrocyte porphyrins(11). The uroporphyrin present in the combined aqueous fractions was adsorbed on a column of aluminum oxide and eluted with 1.5 N hydrochloric acid for fluorimetric analysis.†

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† Different lots of Al_2O_3, from the same company may differ in their suitability for this analysis. Each lot should therefore be tested with pure uroporphyrin for quantitative recovery.

As noted in the following, small amounts of several other unidentified porphyrins are included in the proto-, copro- or uro- fractions as described above. With methods now available it is not feasible to attempt complete porphyrin fractionation in the routine analyses. No satisfactory methods are available for quantitative estimation of the individual non-fluorescing porphyrin precursors which (unlike PBG) are converted to porphyrin by iodine and/or ultra-violet light(10). The following two methods were employed for estimating the total amount of these compounds in aliquots of the primary ethyl acetate:acetic acid extract: (1) Determination of difference in fluorescence intensity of this extract before and after addition of iodine and exposure to ultra-violet light, and (2) determination of fluorescence intensity of 3 N hydrochloric acid extracts of 2 portions of the ethyl acetate, only one of which had been treated with iodine. These procedures were carried out in a dimly-lit room. For more precise analysis of the porphyrins, several of the fractions were esterified with methanol: sulfuric acid (19:1) and further purified by chromatography on calcium carbonate(12). The ratio of coproporphyrins I and III was studied by a slight modification of the previously described fluorescence quenching technic(12) and by the paper chromatographic method of Chu *et al.*(13). Copro-, and protoporphyrins were crystallized as methyl esters from methyl alcohol. Melting points were taken with a Fisher-Johns micro-melting point apparatus. The uroporphyrin fraction was further analyzed by the paper chromatographic method of Falk and Benson(14). A method later devised for quantitative estimation of PBG in the liver(8) was not available at the time the present studies were carried out. Instead, qualitative Ehrlich reactions were observed on 10% acetic acid or 1% ammonium hydroxide extracts of the homogenates. The following method was employed to determine the relative activity of cell-free versus cell-containing fractions of the liver homogenate. From a total of 360 ml, four 25 ml portions were incubated for varying periods after addition to each of 1.0 mg of PBG in 1 ml phosphate buffer pH 7.4. The remaining 260 ml was centrifuged at 3000 rpm for 20 minutes. 160 ml of opalescent cell-free supernatant solutions were removed, and the same volume of phosphate buffer containing penicillin and dihydrostreptomycin was added to the centrifugate. The latter was then mixed by homogenizing for 30 seconds. 25 ml portions of supernate and reconstituted centrifugate were incubated with PBG in the same way as described for the whole homogenate.

Results. The conversion of PBG to porphyrins by normal rat liver homogenate has been studied in 4 separate experiments. In each, considerable amounts of copro- and protoporphyrin and smaller amounts of uroporphyrin appeared. PBG disappeared progressively from the samples, being present in trace amounts at 9 hours and absent at 22 hours. The data from 2 of these studies are summarized in Table I. In a third study analyses were made only at 0, 3 and 6 hours. The 6 hour sample contained 0.4 μg of uroporphyrin, 5.7 μg of coproporphyrin, and 11.6 μg of protoporphyrin per gram of liver following addition of 200 μg of PBG/gram of liver in the homogenate.

Activity of whole liver homogenate as compared with that of the supernatant solution and centrifugate may be seen in Table II. It is evident that the amounts of porphyrin were similar with whole homogenate and supernatant solution. Relatively little conversion to copro- and protoporphyrin occurred in the centrifugate. As in the previous studies, the 9 hour uroporphyrin values exceeded the 22 hour value.

Evidence was regularly obtained for the presence of non-PBG precursors converted to porphyrin by iodine and ultra-violet light. Measurements of fluorescence intensity of the primary ethyl acetate:acetic acid extracts of the homogenates and supernates incubated for 3 to 9 hours showed 3-fold increases or more after the addition of I_2 and exposure to ultra-violet light, the gross fluorescence changing from greenish-yellow to orange or bright red. No increase was observed in the extracts from the similarly treated centrifugates. It is thus evident that over half of the total porphyrin was present in precursor form in these

TABLE I. Conversion of Porphobilinogen to Porphyrin by Normal Rat Liver Homogenate.

Incubation time (hr)	Total porphyrin recovered (μg/g liver)					
	Exp. 1			Exp. 2		
	"Uro"*	"Copro"*	"Proto"*	"Uro"*	"Copro"*	"Proto"*
0	.02	.14	.17	.2	3.3	.8
.5	.02	.4	1.9			
1.2	.08	1.25	2.7			
2	.25	5.0	5.0			
3				1.25	10.0	6.5
4	1.1	11	7.5			
9	3.5	23	19	2.9	25	16
22	1.3	36	34	.8	38	58

0.20 mg porphobilinogen added/g of liver in each series. Control samples incubated similarly without added porphobilinogen all contained less than 0.02 μg of uroporphyrin and 0.06-0.15 μg of coproporphyrin/g of liver. Protoporphyrin values in control samples rose progressively with incubation from 0.06 to 1.2 and from 0.3 to 0.8 μg/g of liver in series 1 and 2 respectively. Total recovery as porphyrin at 22 hr = 51 and 67% respectively. (Values corrected for individual molecular weights.)

* As discussed under *Methods,* it is recognized that this fractionation does not exclude small amounts of other unidentified porphyrins. This also applies to data in Tables II and III.

homogenates and supernates. (In other studies, extraction of the homogenate in the dark has shown over 90% of the porphyrin to be present in the non-fluorescent state.)

In the 2 instances (Tables I and II) in which the significant amounts of coproporphyrin were found in the "0" time samples, most of the porphyrin was present in precursor form. This suggests that significant conversion of PBG had occurred before the sample was deep frozen or during warming to room temperatures.

In addition to control data for liver alone, shown in Tables I and II, the incubation of 0.74 mg of PBG with phosphate buffer and antibiotics alone, yielded 3 μg of uroporphyrin at 9 hours and 72 μg at 22 hours. The latter value is much larger than any of the values noted for incubated homogenate. No copro- or protoporphyrin was demonstrated in this experiment at any period. Only faint traces of porphyrin (less than 0.1 μg total) were found even at 22 hours in the control samples of diluted rat blood without PBG

TABLE II. Conversion of Porphobilinogen to Porphyrinogen and Porphyrin by Rat Liver Homogenate, Supernatant "Solution," and Centrifugate.

Sample	Incubation (hr)	Fluor. increase* (%)	"Uro"	"Copro"	"Proto"
			(μg)		
Homogenate	0	105	.2	10	3
	4	127	.5	63	15
	8	216	19	167	46
	22	45	12	270	105
Supernate	0	0	.4	4	2
	4	79	2	87	28
	8	86	10	159	67
	22	0	4	99	130
Centrifugate	0	0	.1	.3	.2
	4	0	1	13	12
	8	0	8	27	29
	22	0	43	42	41

0.2 mg crystalline porphobilinogen added/g of liver. Control uro- and coproporhpyrin values/g of liver all less than 0.02 μg; control 22 hr protoporphyrin in homogenate = 4 μg and 1.5 to 1.7 μg in other 22 hr samples. Total recovery as porphyrin in 22 hr homogenate = 52%.

* Measurement of porphyrinogen content by comparison of red fluorescence in primary ethyl acetate:acetic acid extract before and after addition of I_2 and brief exposure to ultraviolet light.

These data were presented in somewhat different form at the Ciba Conference, Ref. 15 (p. 211).

while in similar samples containing PBG only uroporphyrin increased significantly (8 μg per mg of added PBG, in 22 hours).

Porphyrins extracted from individual homogenates incubated with PBG were pooled for further analysis in 4 separate batches, depending upon duration of the incubation period. They were esterified and then purified by calcium carbonate chromatography. Chromatography of the "uroporphyrin" fraction yielded one major zone, with only a trace of a second. The "coproporphyrin" fraction yielded from 6 to 8 zones in the 9 to 22 hour samples. Coproporphyrin comprised an average of 74% (range 63 to 85%) of the total of these samples. Six zones were obtained from each of the "protoporphyrin" fractions. A quantitative fractionation in 2 instances revealed that the protoporphyrin comprised but 68 to 69% of the total porphyrin. Paper chromatographic analyses of the proto-, copro-, and uro- fractions revealed the presence of a total of at least 11 different porphyrins, the major components behaving as copro- and protoporphyrin. The behavior of the other fractions indicated the presence of uroporphyrin and of porphyrins with 3, 5, 6 and 7 carboxyl groups.

Isomer studies of the total coproporphyrins pooled from the various incubations revealed 75% to be type III at 9 hours and 58% at 22 hours by the fluorescence quenching technic. The major portion in both behaved as coproporphyrin III by the Chu method.

For crystallization, the combined coproporphyrin ester was evaporated to dryness and the residue was washed twice with 3 ml portions of ether cooled to –15°. The ether soluble porphyrin was crystallized from methyl alcohol, yielding rosettes typical of coproporphyrin III methyl ester, with a melting point of 144-145°. Small curved needles similar to coproporphyrin I were obtained on one occasion from the ether-insoluble residue, but the amount was too small for recrystallization and melting point determination. The combined protoporphyrin methyl ester was crystallized, m.p. 222°C, (proto-9-methyl ester). Analysis of the uroporphyrin by paper chromatography revealed a predominance of type III isomer in samples incubated for 9 hours, whereas approximately equal amounts of types I and III were present in the 22 hour samples.

TABLE III. Conversion of Porphobilinogen to Porphyrins in Chicken Red Blood Hemolysate.

	Total porphyrin recovered (μg)		
Incubation time (hr)	Uro-porphyrin	Copro-porphyrin	Proto-porphyrin
.0	.1	.1	.3
7.3	82	40	30
21.0	94	81	45

25 ml of heparinized chicken blood were lysed with 220 ml H_2O, and 20 ml of 0.1 M phosphate buffer and antibiotics (as for liver homogenates) were added. 500 μg porphobilinogen were added to each flask containing 20 ml of lysed blood. Total recovery as porphyrin = 53% in 22 hr sample; recovery as copro- plus protoporphyrin = 35%.

In a final experiment a study was made of the conversion of PBG to porphyrins in chicken blood hemolysate. The results are summarized in Table III. It will be noted that while the total porphyrin recovery compared well with that obtained with liver homogenates, a much higher proportion was recovered as uroporphyrin when chicken hemolysate was used. Again, significant amounts of porphyrin precursors were noted in the incubated samples.

Discussion. The present study shows that PBG is readily converted to uro-, copro-, proto-, and other unidentified porphyrins by normal rat liver homogenate. Some implications of this finding will be discussed in greater detail in the companion paper(8) and elsewhere. Here we will consider only the relationship of these studies to the problems of heme synthesis.

Various investigators have postulated the biological occurrence of progressive decarboxylation of the 8-carboxyl uroporphyrin via the 4-carboxyl coproporphyrin to the 2-carboxyl protoporphyrin. Considerable evidence against this concept has been presented by one of us(15). The demonstration in the present studies of large amounts of precursors converted to porphyrin by iodine suggested that these presumably reduced porphyrins may be the biologically active intermediates in heme synthesis. Since completion of these studies, reports by Neve and co-workers (16, 17), Granick(18) and Bashour(19) and collaborators have shown that this is indeed the case.

The differing solubility properties of uro- and coproporphyrin and their respective porphyrinogens has permitted their separation and direct identification. To date this has not been possible in the case of protoporphyrin. Evidence for the occurrence of reduced protoporphyrin as a metabolic intermediate is therefore indirect only, and is derived from the fact that in some studies the amount of protoporphyrin isolated after addition of iodine exceeded the total amount of porphyrin present prior to treatment with iodine.

Studies have been made of infra-red spectra and other properties of the various porphyrins isolated from the incubated samples. These are being compared with those of porphyrins obtained by the partial decarboxylation of uroporphyrins I and III and will be reported separately.

Summary. 1. Crystalline porphobilinogen (PBG) incubated with rat liver homogenate has been shown to be converted to at least 11 different porphyrins, the majority of which is copro- and protoporphyrin, with a smaller proportion of uroporphyrin. The total amount at 22 hours incubation in each of 3 experiments was slightly in excess of 50% of the PBG added. 2. At least half of the porphyrin in the 3 and 9-hour incubated samples were present in a non-fluorescing form. These porphyrin precursors were readily converted to porphyrin by addition of iodine or exposure to ultra-violet light. 3. The major part of the porphyrins isolated was isomer type III. The proportion of type I isomer increased with continued incubation. 4. The supernatant suspension obtained by centrifugation at 3000 rpm for 20 minutes was essentially as active as the whole homogenate, in the conversion of PBG to porphyrin.

We are indebted to M. Keprios and M. Goblirsch for their technical assistance, to Miss Irene Bossenmaier who assisted with isolation of crystalline PBG and to Dr. Marie Berg who carried out the paper chromatographic studies.

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Conversion of Porphobilinogen to Porphyrin by Liver Homogenates of Rats with Experimental Hepatic Porphyria.* (23172)

Alphonso Merchante,† Bernardo Leo Wajchenberg,‡ and Samuel Schwartz
Department of Medicine, University of Minnesota Hospitals, Minneapolis.

The companion paper(1) discussed the metabolic role of porphobilinogen (PBG) and its ready conversion to uro-, copro-, proto-, and other porphyrins by normal rat liver homogenate. PBG is excreted in large amount by patients with acute intermittent porphyria and by rats and rabbits with experimental porphyria induced by allyl-isopropyl-acetyl-carbamide (Sedormid)(2). It is believed that this abnormality in PBG and porphyrin metabolism is related primarily to a disturbance in liver metabolism(2,3), and there are two possibilities which might account for the excessive PBG excretion: (1) the liver in these conditions is unable to metabolize PBG at the normal rate, or (2) the production of PBG is increased beyond the capacity of liver to metabolize it.

To investigate these possibilities, studies similar to those previously described for normal rat liver(1) were carried out with livers of rats treated for various time intervals with allyl-isopropyl-acetyl-carbamide.§ A quantitative procedure was developed for the analysis of hepatic PBG and its rate of disappearance from liver homogenates was studied. The incubation data thus obtained were correlated with data on the accumulation of PBG and porphyrin in the livers of treated animals as described below.

Material and methods. Adult male rats were fed allyl-isopropyl-acetyl-carbamide at 100 mg/day as described previously(2). Actual measured intake in individual rats averaged from 70 to 100 mg/day. Preparation of homogenates and methods employed for quantitative analysis of porphyrins have been described(1). The homogenates were incubated without shaking unless noted otherwise. Because the volumes of liver homogenate employed in the different studies varied from 7 to 25 ml, the data are reported in terms of μg PBG or porphyrin/g of liver in the homogenate.

(1) *Quantitative determination of PBG in rat liver homogenate.* Various preliminary procedures employing dilute acetic, citric, or hydrochloric acids, ammonia and sodium hydroxide were discarded because of turbidity obtained on subsequent addition of Ehrlich's reagent with or without sodium acetate, or because of poor recovery. The following procedure, however, yielded clear solutions with reproducible recovery of added PBG.‖ Five ml of 7.5% trichloracetic acid were added to 2.5 ml of liver homogenate to yield a final concentration of 5% trichloracetic acid. The well mixed suspension was shaken for several minutes and centrifuged. To 2.5 ml of supernatant solution were then added 7.5 ml of Ehrlich's reagent prepared as recommended by Valquist(4).¶ The absorption of this solution was determined with an Evelyn colorimeter employing a 565 mμ filter. Because the color is somewhat unstable, the maximum absorption reading, obtained within 1 minute, was used in all cases for quantitative analysis.

In two series of recovery experiments solutions of different batches of PBG crystals were added to different liver homogenates, extracted with trichloracetic acid, and analyzed as described above. Results are shown in A Fig. 1. Curve B was obtained by diluting solutions of PBG directly with 5% trichlor-

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† Fellow, Doherty Foundation (N.Y.) Present address: Medical School, Madrid, Spain.

‡ Eli Lilly Fellow from University of Sao Paulo Medical School, Brazil.

§ We are indebted to Hoffmann-LaRoche, Inc., Nutley, N. J. for generous supply of this material.

‖ The possible use of such a method was discussed by one of us (S.S.) with Dr. Robert Aldrich, University of Oregon, in the summer of 1953. His results with a similar procedure are in general agreement with those reported here (personal communication).

¶ 20 g *p*-dimethylaminobenzaldehyde dissolved in 500 ml conc. HCl plus 500 ml H_2O.

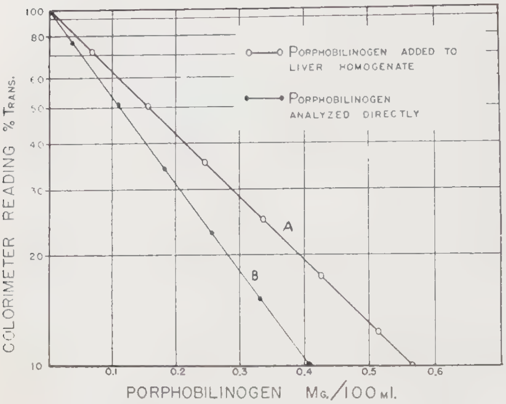


FIG. 1. Colorimetric estimation of porphobilinogen. A: Porphobilinogen added to homogenate of normal liver. B: Porphobilinogen added to 5% trichloracetic acid and analyzed directly. Readings taken with Evelyn Colorimeter, employing a 565 mμ filter.

acetic acid (without liver homogenate) prior to addition to Ehrlich's reagent. A consistent difference was found between the 2 curves at concentration levels tested. The reason for this has not been established. Curve A was employed for the analyses reported herein.

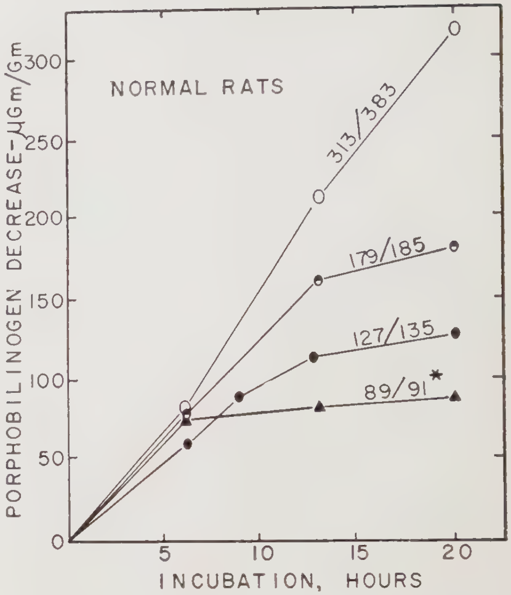
(2) *Estimation of hepatic PBG in rats treated with allyl-isopropyl-acetyl-carbamide.* Groups of 3-5 rats were fed allyl-isopropyl-acetyl-carbamide for periods up to 13 days. As shown in Table I no detectable PBG was present during the first 3 days. In general, increased amounts were found from the fifth to thirteenth days though values varied considerably in individual rats.

(3) *Fate of PBG incubated with homogenates of livers from normal and from "porphyric" rats.* a. Disappearance of PBG. Groups of 5 rats each were fed allyl-isopro-

TABLE I. Concentration of Liver Porphobilinogen in Rats Fed Allyl-Isopropyl-Acetyl-Carbamide.

Feeding period, days	Porphobilinogen, μg/g	
	Range	Mean
0	0– 0	0
2	*	0
3	0– 0	0
5	*	115
6	*	98
7	0–168	89
10	12–234	122
13	*	227

* 5 rat livers pooled for assay. 3 to 5 individual livers analyzed at other time intervals.



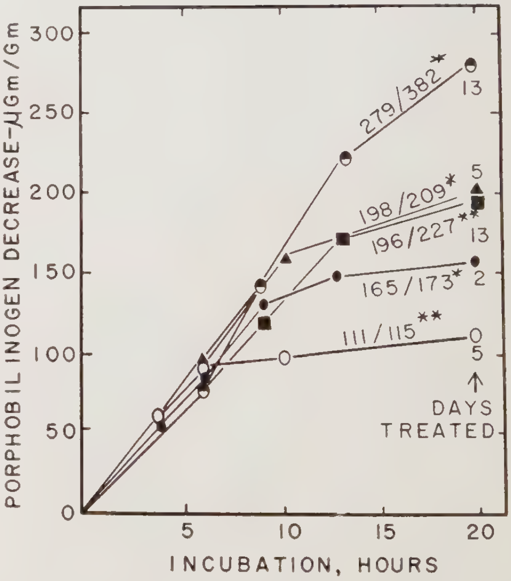


FIG. 2 (top). Disappearance of porphobilinogen from incubated liver homogenates from normal rats. Numerator = total μg of porphobilinogen disappearing from homogenate/g of liver during 20 hr incubation; denominator = μg of porphobilinogen added to homogenate/g of liver at 0 time.

FIG. 3 (bottom). Disappearance of porphobilinogen from incubated liver homogenates from Sedormid-treated rats. Homogenates incubated both with (*) and without (**) added porphobilinogen. Ratios again refer to μg porphobilinogen disappearing in 20 hr/μg present at 0 time/g of liver.

TABLE II. Porphyrin Content of Liver Homogenates Incubated with Porphobilinogen.

		Porphyrin, μg/g Incubation period					
Sedormid feeding (days)	Porphobilinogen, μg/g*	6 hr		13 hr		21 hr	
		"U"	"C + P"	"U"	"C + P"	"U"	"C + P"
0	91	9	33	4	29	13	14
0	185	7	17	26	45	9	20
0	383	4	20	23	24	34	27
2	173	7	27	1	29	1	14
5*	115	4	25			6	19
13*	227	9	29	13	33	17	16
13	382	8	27	16	21	23	19

* Porphobilinogen content of unincubated sample; solution of crystalline porphobilinogen added to all homogenates prior to incubation except for 5- and 13-day fed rats.

"U"—uroporphyrin fraction. "C + P"—copro- + protoporphyrin fraction.

pyl-acetyl-carbamide for 2, 5 and 13 days respectively. The livers from each group were combined for homogenization. The homogenates were incubated for various periods to 20 hours both with and without added PBG. With each group of treated rats, combined livers from 4 control untreated rats were analyzed similarly. In addition, varying amounts of PBG were added to 4 aliquots of the combined homogenate of a fourth group of normal rats. Data summarizing the decrease in PBG concentration of incubated homogenates of normal and Sedormid-treated rats are shown in Fig. 2 and 3. The same amount of PBG disappeared from the homogenate/g of liver during the first 6 hours, regardless of the initial PBG concentration. At this time interval 60 to 91 μg (average 73 μg) disappeared/g of liver in the 7 studies employing normal rats, while the corresponding values for Sedormid-treated rat livers ranged from 70 to 90 μg (average 81 μg).

Porphyrin analyses of several of the homogenates described in Fig. 2 and 3 are summarized in Table II. These data, too, indicate similar patterns of porphyrin accumulation in liver homogenates of normal(1) and Sedormid-treated rats; essentially similar concentrations of porphyrin were found during the first 6 hours in each sample. In most instances the recovered copro-plus protoporphyrin decreased in amount between 13th and 21st hours. In possible contrast to uroporphyrin(5), the latter porphyrins require enzymatic activity for formation from PBG and their presence is therefore especially significant. In agreement with the data reported in the companion paper, the major fraction of coproporphyrin behaved as the type III isomer on paper chromatographic analysis. After 25 hours incubation the uroporphyrin behaved chiefly as type I isomer.

In a final experiment, the effect of increased aeration on porphyrin content of incubated homogenates was investigated. Constant shaking resulted in the finding of increased amounts of uro-type porphyrin associated with a marked reduction in recovery of porphyrin in the coproporphyrin fraction. Shaking had no significant effect on the rate of porphobilinogen disappearance in at least 2 of the 3 studies. The data at 12 and 20 hours are summarized in Table III.

Discussion. It is evident that, under the conditions employed, the liver of Sedormid-treated rat is able to metabolize PBG at least as readily as the normal rat liver. The production of porphyrin from PBG was likewise similar in both. It would therefore appear that the excessive amounts of PBG in the liver and urine of these animals depends simply on an overproduction.

The nature of the primary abnormality in Sedormid-treated animals remains uncertain. Labbe and co-workers(6) have postulated a block in conversion of delta amino levulinic acid to purine metabolites(7), with resulting overproduction of pyrrol compounds. If this mechanism is significant it must involve only certain specific purines since we have found no significant alteration in concentration of ribo- or desoxyribonucleic acids in livers of Sedormid-treated rats, though there is a sig-

TABLE III. Effect of Aeration on Porphyrin Content of Liver Homogenates Incubated with Porphobilinogen.

Sedormid feeding (days)	Sample*	Porphobilinogen, µg/g		Porphyrin, µg/g Incubation period 12 hr			20 hr		
		0 time†	12 hr	Uro	Copro	Proto	Uro	Copro	Proto
0‡	A	117	33	4	20	25	8	1	25
	B	"	41	17	0.4	23	21	2	3
6	A	98	16	5	22	40	8	2	51
	B	"	15	24	0	36	17	0	34
6‡	A	211	114	14	14	29	17	4	59
	B	"	78	20	0	37	33		

Livers of 6 normal rats and 6 Sedormid-treated rats pooled.

* Sample A, no shaking. Sample B, continuous shaking.

† Porphobilinogen conc. in unincubated sample.

‡ Solution of crystalline porphobilinogen added to homogenate.

nificant decrease in concentration of total organic acid soluble phosphorus. It is also not clear how such a concept would explain the rapid decrease in liver catalase(8) and accumulation of large amounts of green porphyrins as recently described(9), the latter found within a few hours after feeding Sedormid or administering allyl-isopropyl-acetamide intraperitoneally to rats.

If the increased production of PBG is intimately related to or is the primary defect in (Sedormid) porphyria, one might expect to detect this phenomenon during the first few hours after Sedormid feeding. Thus far this has not been possible. Such detection would now seem to require either more sensitive analytical technics, or isotopic studies to determine the size of the hepatic PBG "pool." Meanwhile, the delayed appearance of PBG in liver and urine after Sedormid feeding, is consistent with either of the following concepts: (1) Production of PBG in the early period may actually be increased, but only to levels which can be completely metabolized, or (2) the increase in PBG production may occur only after several days as a secondary compensatory phenomenon, being stimulated by decreasing concentration of catalase and perhaps of other heme enzymes. This situation would be analogous to the stimulated synthesis of hemoglobin which follows excessive blood loss.

Summary. Liver homogenates of rats treated with allyl-isopropyl-acetyl-carbamide (Sedormid) converted PBG to porphyrin at approximately the same rate as homogenates of normal rat liver. It is concluded that the excess PBG in the liver and urine of the "porphyric" rats is due to overproduction and not to a block in its further metabolism.

We are indebted to Dr. C. J. Watson and Miss Irene Bossenmaier for the crystalline PBG used, and to Dr. Marie Berg for paper chromatographic analysis of the partially purified porphyrins.

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Serum Arginase Activity.* (23173)

ROBERT T. MANNING† AND SANTIAGO GRISOLIA‡
(Introduced by G. L. Curran)
Department of Medicine, University of Kansas School of Medicine, Kansas City, Kan.

Assays for various serum enzymes, such as transaminase(1) and aldolase(2) have been utilized to detect hepatocellular necrosis or damage. These enzymes, however, are present in all tissues, and gross alterations in their serum activities are not specific for liver damage. Since arginase is present in significant quantity only in liver(3), we have explored the usefulness of measuring activity of this enzyme in normal sera and in the sera of patients with liver disease.

Method. Blood samples are collected in unoxalated tubes and the serum separated and frozen until assay. One part of serum (usually 2 ml) is mixed with 2 parts saturated ammonium sulfate (pH 9) and centrifuged at 4000 x g for 15 minutes at 0°C. The supernatant fluid is discarded. The protein precipitate is taken up in saline and the ammonium sulfate precipitation repeated. This procedure removes the bulk of the urea present in the serum. The assay for arginase is carried out routinely by incubating in centrifuge tubes at 38°C for 30 minutes the twice ammonium sulfate precipitated proteins of the serum (usually the equivalent of 1 ml of serum) with the following components in a final volume of 2 ml: 0.5 ml of 0.2 M maleate buffer at pH 7.0(4) (containing 0.05 M $MnCl_2$) and 0.5 ml of 1.14 M L-arginine, adjusted to pH 9.4. The pH of the final mixture is 9.0. A blank without serum is included as well as blanks without arginine for the estimation of endogenous urea. Activity is proportional to enzyme concentration provided that no more than 1% of the substrate has been utilized(4). The reaction mixture is deproteinized by the addition of 2 ml of 1 M $HClO_4$. The tubes are centrifuged at 2000 x g for 10 minutes and aliquots of the protein-free supernatant fluid are analyzed for urea by the method of Archibald(5) with the following modifications: Aliquots containing no more than 1 μmole of urea (usually 2 ml) are made up to 5 ml and 2 ml of a 3 to 1 mixture H_2SO_4-H_3PO_4 mixture (technical H_2SO_4 should be used—presumably due to the requirement for trace metals(6,7)) and 0.4 ml of isonitrosopropriophenone (4% in ethanol) are added. The contents are mixed, the tubes capped with rubber stoppers fitted with capillary tubes, and heated in a covered, boiling water bath for one hour. After cooling in the dark (the color is light sensitive and should be protected from direct sunlight), a reading at 540 mμ is obtained. Since color development deviates from Beer's Law, a calibration curve is necessary.

Results. Table I records the mean and standard deviation of the arginase activity obtained in sera of 34 healthy blood donors. Table II records the values obtained in patients with liver damage. Values are recorded in μmoles of urea per ml serum per 30 minutes incubation at 38°C.

Discussion. Removal of the urea normally present in the serum by ammonium sulfate precipitation is not complete but the residuum is small. That control tubes should be run is emphasized by inspection of the experiment with serum from a patient with high blood urea level (Case No. 17; blood urea 210 mg %) which would have given erroneous results without control tubes. It is apparent from the tables that there is considerable variation in the blank values. Whether this is due to intrinsic variations in these

TABLE I. Serum Arginase Determinations in 34 Healthy Blood Donors.

		Mean	μM urea		
♂	♀	age	Control tube	Assay tube	2 − 1
29	5	28.5	.10 ± .07	.14 ± .16	.06 ± .09

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† Trainee, Nat. Inst. of Arthritis and Metabolism.

‡ Established Investigator, Amer. Heart Assn.

TABLE II. Serum Arginase Determinations in 19 Patients with Liver Disease.

			μM urea			
No.	Age	Sex	Control tube	Assay tube	2 − 1	Diagnosis
1	19	♂	.00	.72	.72	Infectious hepatitis
2	40	♂	.00	1.98	1.98	Laennec's cirrhosis§
3	40	♂	.18	1.02	.84	*Idem* §
4	52	♂	.20	.89	.69	”
5	35	♀	.13	.19	.06	? ”
6	35*	♀	.00	1.20	1.20	” §
7	35†	♀	.00	1.36	1.36	” §
8	47	♀	.03	.39	.36	”
9	38	♀	.42	1.56	1.14	”
(10)	33	♀	.18	.02	.00	? ”
11	19‡	♂	.24	.10	.00	Serum hepatitis‡
12	50	♀	.12	.65	.53	Post-necrotic cirrhosis
13	50	♀	.06	2.38	2.32	*Idem*
14	45	♀	.39	6.47	6.08	Cardiac cirrhosis
15	45	♀	.45	5.95	5.50	*Idem*
16	45	♀	.42	3.08	2.66	”
(17)	61	♂	.85	1.09	.24	Hepatoma§
18	48	♂	.28	.43	.15	Fatty infiltration
19	38	♀	.24	.20	.00	Hepatic coma (etiology ?)
	Mean		.22 ± .23	1.56	1.36	

* Simultaneous peripheral vein sample. † Hepatic vein wedge sample. ‡ Recovery phase. § Confirmed by autopsy or biopsy.

samples, as for example higher endogenous urea or to other chromogenic materials which are occluded during the ammonium sulfate precipitation, is not known.

Case No. 10 represents a patient with clinically suspected liver disease which was not confirmed by laboratory studies. The arginase activity was zero in this case.

Assay of ornithine formation has not proved technically feasible for routine assay.

Summary. Serum arginase activity has been determined in 34 healthy blood donors. A mean value 0.06 ± 0.09 μmole urea formed/ml serum was found with a range of .00 to 0.36 μmole. In sera of 19 patients with proven or suspected liver disease the mean arginase activity was 1.36 with a range of .00 to 6.08 μmoles formed/ml serum under the condition of assay.

We wish to thank Dr. Mahlon Delp for helpful discussion and for access to the clinical material.

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Influence of Sex on Adrenal Regeneration and Accompanying Hypertension.
(23174)

A. W. Neff and J. T. Correll (Introduced by M. H. Kuizenga)
Department of Biochemistry, Research Division, The Upjohn Co., Kalamazoo, Mich.

Skelton and Guillebeau(1-3) have reported that rats subjected to right nephrectomy, right adrenalectomy and left adrenal enucleation, demonstrated regeneration of adrenal tissue accompanied by hypertension and characteristic lesions. In this laboratory female rats so prepared (henceforth referred to as NAE-rats) yielded results similar to those reported. However, when a group of male rats was prepared in the same manner only 3 of 31 became hypertensive after 9 weeks of observation. The average systolic blood pressure was 125 mm Hg, ranging from 102 to 164 mm Hg, whereas, females averaged 148 mm Hg, ranging from 140 to 160 mm Hg. Control values averaged around 110 mm Hg. Correspondingly heart weights of the hypertensive females were significantly greater than those of the males. Even more significant was the observation that the hypertensive females had regenerated nearly 3 times more adrenal tissue than the normotensive males.

This suggested that sex hormones might be involved, either directly or indirectly, in adrenal regeneration accompanied by hypertension. Failure to elicit a blood pressure rise in male rats was possibly due to an inhibiting effect of androgens. To explore this possibility, 3 approaches were made. First, NAE-female rats were treated with testosterone to determine whether the male hormone might suppress regeneration of the adrenal and hence the subsequent development of hypertension. Second, NAE-hypertensive female rats were ovariectomized and then given daily injections of testosterone propionate. This study was designed to ascertain if an established hypertension could be reversed by radical treatment, *i.e.*, a minimum of estrogen plus an excess of androgen. Third, NAE-males were castrated to reduce endogenous testosterone.

Materials and methods. Rats from the Upjohn colony (Wistar strain), 4 weeks old, weighing 60-65 g were caged individually and maintained on an adequate stock diet and 1% sodium chloride solution *ad libitum*. Beginning at the ninth week and at weekly intervals thereafter, systolic blood pressures were determined using a photoelectric tensometer (4). Following extensive observations among normal and DCA-hypertensive rats it has been the practice in this laboratory to consider pressures above 140 mm Hg as hypertensive. All the animals were sacrificed at 17 weeks of age, necropsied, various organs weighed, and the adrenal glands saved for histologic study. The experimental groups were as follows: *Group I.* 10 female rats as untreated unoperated controls. *Group II.* 10 NAE-female rats were prepared. Starting immediately post-operatively, testosterone propionate (TP) at a dose of 100 μg/rat/day was administered subcutaneously. Six weeks post-operatively the dose was increased to 200 μg. *Group III.* Eighteen NAE-female rats were prepared. Eight weeks post-operatively, when hypertension had developed, this group was divided so that the 2 sub-groups of 9 rats each had the same average blood pressure. One sub-group served as hypertensive controls, the other constituted Group IV. *Group IV.* Nine hypertensive rats from Group III were ovariectomized and injected subcutaneously with 200 μg of TP/rat/day. *Group V.* Fifteen males were castrated 3 days before NAE treatment. *Group VI.* Five males castrated only and carried as controls. *Group VII.* Fifteen NAE-male rats were prepared. *Group VIII.* Five male rats were maintained as unoperated controls.

Results. Average systolic blood pressure values over the 13 week experimental period for each group are presented graphically in Fig. 1. In agreement with previous experiments and published reports(1,2), it was found that NAE-female rats (Group III) developed high blood pressure as compared to

TABLE I. Terminal Data on NAE-Prepared Rats Subjected to Various Treatments.

Group No.	No. of rats	Description of treatment	Final blood pressure (mm Hg)	Body wt (g)	Heart	Adrenal
					(mg/100 g body wt)	
I	10	Unoperated ♀ controls	106 ± 2	239 ± 6*	315 ± 4	20.6 ± .8†
II	10	NAE-♀ + TP	123 ± 3	281 ± 4	329 ±10	7.8 ± .5
III	9	NAE-♀	150 ± 3	246 ± 8	370 ± 7	16.7 ±1.0
IV	8	NAE-♀ + ovariectomy + TP	129 ± 2	260 ± 5	350 ± 7	12.6 ±1.0
V	15	Castrated + NAE-♂	114 ± 3	301 ± 6	281 ± 6	6.5 ± .3
VI	5	Castrated ♂ controls	109 ± 2	332 ±13	263 ± 8	10.6 ± .7†
VII	15	NAE-♂	124 ± 3	385 ± 6	270 ± 9	5.7 ± .5
VIII	5	Unoperated ♂ controls	107 ± 2	391 ±14	258 ± 2	10.4 ± .3†

* Stand. error of the mean. † Both adrenals.

TP = Testosterone propionate. NAE = Right nephrectomy, right adrenalectomy and left adrenal enucleation.

intact female controls (Group I). In contrast, NAE-male rats (Group VII) had significantly ($P<0.01$) lower blood pressures throughout the experimental period than those of the NAE-females (Group III) and only slightly greater than those of the controls (Groups I and VIII).

From the data presented in Table I, it was seen that heart weights generally correlated with terminal blood pressure values. Terminal adrenal weights confirmed the previous observation that those NAE-rats which developed higher blood pressures also demonstrated the most regeneration of adrenal tissue. Thus, the NAE-female rats (Group III) had single adrenals which weighed nearly as much as both adrenals from the intact females (Group I). With either the NAE-males (Group VII) or the castrated NAE-males (Group V) there appeared to be some adrenal regeneration as the single adrenals had terminal weights equal to about half of the weights of 2 adrenals from the controls (Groups VIII and VI). Histologic examination indicated that limited regeneration had taken place, largely, in the *zona fasciculata*. Blood pressure increases in these groups (Groups V and VII) were correspondingly slight.

Daily administration of testosterone propionate to NAE-female rats (Fig. 1, Group II) had a moderating effect on development of higher blood pressures for 5-6 weeks postoperatively at which time the pressure differences of treated and untreated rats became insignificant. Upon increasing the dose of TP 2-fold the systolic blood pressure of the treated group was again depressed significantly ($P<0.01$) below the untreated group (Group III) for the duration of the experiment. The smaller average heart weight (Table I) of the TP-treated rats (Group II) confirmed the blood pressure observations; regeneration of adrenal tissue was found to be less than half that of the untreated NAE-females (Group III).

NAE-females which had become hypertensive and then subjected to ovariectomy plus daily TP administration for 30 days (Group IV), demonstrated a significant fall in blood pressure (Fig. 1). Other data for this group (Table I) revealed that terminal adrenal weight was statistically smaller than that of

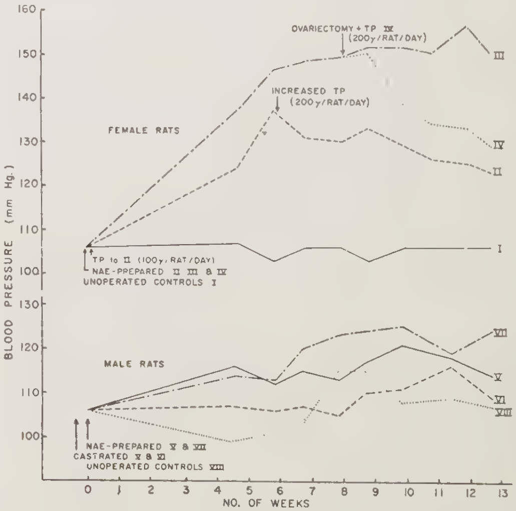

FIG. 1. Systolic blood pressure of NAE-male and female rats.

the untreated controls (Group III) ($P < 0.01$); the enlarged, hypertensive heart had not regressed significantly.

Discussion. Testosterone treatment accompanied with ovariectomy not only reversed an established hypertension of NAE-female rats but also appeared to cause a regression of adrenal mass (Group IV), since, at the time treatment was initiated, the adrenals of Groups III and IV were presumably equal and close to maximal size. It has been reported(5) that testosterone provoked adrenal atrophy when administered to female rats under other experimental conditions. However, reduction of endogenous testosterone by castration of NAE-male rats did not produce high blood pressure even though all NAE-males showed some adrenal regeneration. It is possible this phenomenon is dependent upon the presence of an adequate concentration of estrogens. Estrogens have been reported to produce adrenal hypertrophy (6) and hypertension in rats(7,8). The response of the castrated NAE-male rat to exogenous estrogen is being investigated.

Summary. Evidence has been presented that NAE-female rats but not NAE-males demonstrated adrenal regeneration accompanied with hypertension. Testosterone administered to NAE-female rats inhibited regeneration of the enucleated adrenal and development of hypertension. Testosterone caused a reduction in the blood pressure of ovariectomized, hypertensive NAE-female rats. Castration was not effective in promoting adrenal regeneration or hypertension in the male rat.

The authors wish to thank the following members of The Upjohn Co.: Mr. S. C. Lyster for performing the ovariectomies. Dr. H. D. Webster for histological studies and Mr. F. LaPlante for care of the animals.

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A Method of Isolating Normal and Fowlpox Infected Chorioallantoic Epithelium.* (23175)

CHARLES C. RANDALL AND WILLIAM M. TODD
Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tenn.

In this laboratory we are concerned with an investigation of certain chemical changes which appear to be induced in chorioallantoic epithelium infected with fowlpox virus. Goodpasture(1) has shown that in fowlpox infection the hyperplastic ectoderm contains numerous large inclusions, that to a lesser degree the entoderm is involved as well, and that there is accompanying thickening of the mesoderm. It would be very desirable to obtain infected epithelium in quantity without inclusion of mesodermal elements which may tend to obscure the results.

Procedure. Three different batches each consisting of 3-6 dozen embryonated hen eggs were inoculated with fowlpox on the chorioallantoic membranes by the Goodpasture method(2) and incubated for 5 days at 37°C. A comparable number of uninoculated control eggs were incubated under the same conditions. Infected and control membranes were collected aseptically in cold physiological saline and refrigerated for several hours until

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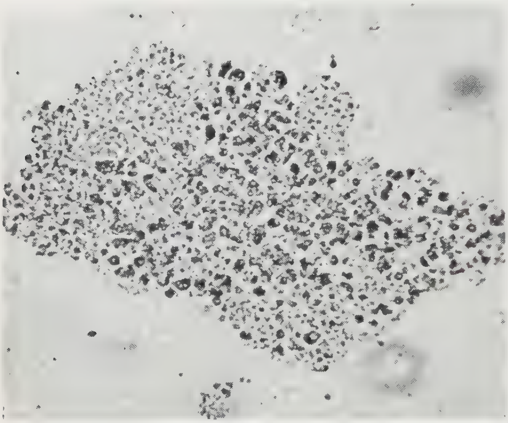

FIG. 1. Unstained wet mount of epithelium of infected chorioallantoic membrane. The dark masses represent intracytoplasmic inclusions. The tissue was teased flat with some tearing, freeing individual inclusions and minute fragments. 120 ×.

ready for use. Ten to 15 membranes at a time were shaken by hand in 10 ml of cold saline with glass beads in a 125 ml rubber stoppered flask for approximately 30-120 seconds. Preliminary experiments indicated that 30 to 60 seconds were adequate for infected tissue whereas normal tissue required longer agitation. The suspension was decanted from the membranes and like preparations were pooled and further diluted with cold saline and allowed to sediment by gravity for 30 minutes to 1 hour. Small fragments rapidly settled out, leaving red cells and debris in suspension. This was aspirated and fresh saline added. This procedure was repeated and the tissue washed 8 to 10 times until the supernatant was crystal clear. Small aliquots were withdrawn for microscopic examination and the remainder centrifuged for 10 minutes at 10,000 rpm in a Servall Angle Centrifuge. The resulting firm pale grey bottom material was frozen and lyophilized. Representative samples of the shaken membranes were fixed in formalin for subsequent histological examination. Several normal and infected membranes were included for comparison and are referred to as control tissue.

Results. Microscopic examination of the clean sedimented material from the infected membranes revealed numerous epithelial fragments varying in size from 0.1 to 2 mm. Red cells were rare and no mesoderm was identified. After study of the fixed and stained control membranes several generalizations as to origin of the epithelium appear justified and are consistent with recorded data(1). Many of the fragments were composed of cells containing typical intracytoplasmic inclusions, characteristic of fowlpox (Fig. 1), and appeared ectodermal in origin. Other pieces of tissue, though hyperplastic, contained few or no inclusions and were probably derived from the entodermal layer. Study of hemotoxylin and eosin stained shaken membranes showed the entoderm to be stripped free in many instances, but present in others, whereas the ectodermal layer was uniformly denuded.

Examination of sediment prepared from normal membranes revealed numerous small fragments of epithelium of mixed ectodermal and entodermal origin, and hemotoxylin and eosin slides of the parent membranes showed considerable desquamation of both cell layers.

Comparison of the lyophilized materials from infected and normal sources consistently revealed that a bulkier and heavier pellet was obtained from the infected membranes. For example, the epithelium obtained from 72 infected eggs weighed 366.7 mg in contrast to 77.9 mg derived from 42 normal eggs. The yield is understandably quite variable since the primary purpose is purity of the preparation.

Discussion. Putnam(3) has pointed out the difficulties attendant on determining chemical changes in tissues infected by viruses because of inflammation and cellular degradation. Our results have shown that the infected tissue in which the specific lesions are numerous can be separated from the mesoderm. This separation is clearly an advantage when chemical determinations are contemplated. The technic described possibly has other uses, as preliminary experiments have shown that the small epithelial clumps are suitable for tissue culture.

Summary. A method is described for isolating chorioallantoic epithelium from normal and fowlpox infected embryonated hen eggs.

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Effect of Acute Liver Damage Plus Hypoxia on Plasma Erythropoietin Content. (23176)

T. C. PRENTICE AND E. A. MIRAND

Roswell Park Memorial Institute, Buffalo, N. Y.

The factors which regulate erythropoiesis are gradually being elucidated. That a circulating humoral factor in the plasma is important in stimulating red cell production under many circumstances is gaining increasing acceptance(1-6). This material has been demonstrated in the plasma from experimental animals and humans with anemias of several different etiologies(7,8), and from several animal species(9-11). A consistent finding has been that the plasma from animals made anemic with phenylhydrazine was more active in stimulating erythropoiesis than the plasma from bled animals(12-14). Some have maintained that degree of anemia was the important factor here, a more severe anemia being produced by phenylhydrazine administration than could be produced by bleeding, with survival of the animal. Recently the observation has been made that this enhanced erythropoietic activity of plasma from phenylhydrazine-anemic animals is in some way related to liver damage(15).

The present study is a further effort to clarify the status of the liver as a controlling influence on the presence and titer of erythropoietic factor in the plasma. During the past year and one-half efforts have been made to demonstrate EPF* in the plasma of normal rats placed in chambers and subjected to atmospheres of 8-12% oxygen for varying periods of time. These results were uniformly negative. At no time were we able to demonstrate any ability of the plasma from these rats kept in this low oxygen atmosphere to stimulate increased Fe^{59} uptake in recipient hypophysectomized or normal rats. It was postulated that perhaps if acute liver damage were induced prior to placing the animals in the chamber an erythropoietic factor might then be demonstrable in their plasma. This proved to be the case and the results are here reported.

Materials and methods. The chambers were constructed of plexiglass. Into each chamber was directed a mixture of nitrogen and oxygen, the proportions of which, along with ambient pressure, determined the simulated altitude. The flow of nitrogen and oxygen was regulated by a rotameter type of flowmeter† and remained quite constant over the experimental period. The chamber O_2 concentration was checked by reading of the oxygen and nitrogen flowmeters and also by a portable Beckman Oximeter. Random air samples were also taken from the chamber in a Douglous bag and checked with a mass spectrograph for O_2, N_2 and CO_2. Good agreement in results was obtained. A flow of approximately 5 liters/minute was maintained through the chamber and soda lime and sulfuric acid were placed to minimize CO_2 and humidity levels.

Ten to 15 normal rats were placed in each chamber and kept there for periods ranging from 48 hours to 5 days. Food (Purina Laboratory Chow) and water were replaced as necessary. At the end of the experimental period the rats were removed, bled from the dorsal aorta and the plasma frozen and stored. A final hematocrit was done on each animal and no animal with

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* Refers to plasma erythropoietic factor.

† Chicago Anesthesia Equipment Co.

TABLE I. Normal and Hypophysectomized Rats that Received Plasma from Hypoxic Animals on 2 Consecutive Days, 1 μc Fe^{59} on Day 3, and 24-Hour Fe^{59} Uptake on Day 4.

*	No. rats	Recipient rats	cc plasma per day	24-hr uptake (%) + 5.0
NP	5	Hypox	2	12.0 ± 4.62
CP	5		2	16.6 ± 9.54
NP	6		2	6.0 ± 4.53
CP	3		2	3.4 ± 1.99
NP	5		2	7.0 ± 7.4
CP	4		2	11.4 ± 10.98
NP	6		2	2.4 ± 2.35
CP	5		2	2.6 ± 1.80
NP	6	Normal	6	53.0 ± 8.40
CP	6		6	50.6 ± 4.98
NP†	10		4	62.6 ± 14.52
CP	9		4	64.6 ± 10.07
NP†	4		6	48.0 ± 10.14
CP	6		6	52.0 ± 3.88

* NP = Normal plasma; CP = Chamber plasma.
† Fe^{59} given on same day as last inj. of plasma.

The "t" test of significance for differences between means was applied to all exp. and control groups and no significant differences existed.

hematocrit below 40 was used as a source of plasma. Acute liver damage was produced in rats by injecting .25 cc CCl_4 subcutaneously just once. These rats were placed immediately in the chamber and left there for 48 hours in an atmosphere of approximately 10% oxygen. They were then withdrawn, exsanguinated from the dorsal aorta and the plasma frozen and stored until used. A final hematocrit was done on each animal and no animal with hematocrit below 40 was used as a source of plasma.

Recipient animals were Sprague-Dawley rats hypophysectomized between the ages of 2 and 3 months and used as test animals approximately 40 days after hypophysectomy. They received 2 cc of plasma I.V. via the jugular vein on 2 successive days. On day 3 approximately 1 μc Fe^{59} was given I.V. via femoral vein. Both the experimental and control groups were then split in half. Half of each group received Fe^{59} 3 hours after the last plasma injection and an 18-hour uptake was done the following day (day 3). The other half received Fe^{59} 24 hours after their last plasma injection, and a 24-hour uptake was done 24 hours thereafter (day 4).

Results. When normal rats were placed in low oxygen atmosphere (8 to 12%) for periods of either 48 hours or 5 days, we could demonstrate no significant stimulating effect of their plasma in either normal or hypophysectomized recipient animals (Table I). However, when acute liver damage‡ was induced with CCl_4 and the animal then placed at the same altitude as the normals for 48 hours, their plasma possessed distinct stimulating properties (Tables II and III). It is apparent by comparison of Table II with Table III that more clearcut differences in hypophysectomized recipients were obtained when Fe^{59} was given at a later period after the last plasma injection and a 24-hour uptake then carried out.

Discussion. The present results suggest that the normal liver inhibits the ability of the normal rat in a hypoxic atmosphere to deliver to or elaborate in its plasma a factor capable of stimulating an increased Fe^{59} uptake in another animal. However, when the liver is damaged acutely with CCl_4 this inhibiting effect is decreased and marked increments of such a factor then appear in the plasma of the hypoxic animal.

Jacobsen *et al.*(15) have demonstrated the importance of liver damage in phenylhydrazine-anemia as a prerequisite to the outpouring of EPF into the plasma. Our findings tend to support these observations and demonstrate that production of significant amounts of EPF can be stimulated in an animal with no anemia provided hypoxic hypoxia and liver damage are present in combination.

The method of demonstrating an erythropoietic response in recipient test animals is a problem in itself. What constitutes reliable evidence of significant erythropoietic stimulation and what does not? A measurement of total red cell volume in each animal with labeled red cells before and after the experiment to detect a significant increase is per-

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‡ Sections of livers show varying degrees of liver damage. This appears as vacuolization of the cells of part or all lobules and necrosis in the centrilobular region. Mitotic figures are present indicating regeneration. The rats subjected to low oxygen tension show a more severe degree of damage than those in normal atmosphere.

TABLE II. Hypophysectomized Rats Given 2 cc of Plasma I.V. on 2 Consecutive Days, 1 μc Fe^{59} I.V. 3 Hr after Last Plasma Injection on Day 2, and 18-Hr Uptake Done on Day 3.

	No. of animals	Avg wt (g) & range	Avg Hct (%) & range	Avg Fe^{59} uptake (%)
Group I—Receiving plasma from rats given .25 cc CCl_4 and kept at 10% oxygen 48 hr	6	198 (172-206)	47 (45-48)	9.9 ± 8.45*
Group II—Receiving plasma from rats given .25 cc CCl_4 and kept at normal oxygen tension 48 hr	7	199 (193-205)	46 (40-48)	1.5 ± .03
Group III—Receiving normal plasma	7	198 (193-208)	45 (43-47)	3.2 ± 1.24
Group IV—Untreated	6	205 (199-221)	50 (46-52)	3.7 ± 3.5

* ± S.E.

The "t" test of significance for differences between the means was applied to group I *vs* groups II, III and IV. Values were significant at 1% level comparing groups I and II. Significance was between 1% and 2% comparing group I *vs* III and IV.

TABLE III. Hypophysectomized Rats Given 2 cc of Plasma I.V. on 2 Consecutive Days, 1 μc Fe^{59} I.V. on Day 3, and 24-Hr Uptake Determined on Day 4.

	No. of animals	Avg wt (g) & range	Avg Hct (%) & range	Avg Fe^{59} uptake (%)
Group I—Receiving plasma from rats given .25 cc CCl_4 and kept at 10% oxygen 48 hr	7	194 (185-205)	44 (43-47)	37.5 ± 3.8 *
Group II—Receiving plasma from rats given .25 cc CCl_4 and kept at normal oxygen tension 48 hr	6	193 (186-217)	47 (45-50)	10.7 ± 2.88
Group III—Receiving normal rat plasma	7	209 (186-218)	45 (43-47)	7.0 ± 4.86
Group IV—Untreated	6	191 (180-203)	48 (45-50)	3.1 ± 1.8

* ± S.E.

The "t" test of significance for differences between means was applied to group I *vs* groups II, III and IV. The difference was significant at the level of less than 1% in all.

haps the most direct assessment of response. Other methods such as 1) response of Hb, Ht, RBC and retic. count 2) marrow cellularity and differential, possess quantitative errors largely due to fluctuations in plasma volume and faulty sampling. The 24-hour Fe^{59} uptake is simple and widely used to demonstrate response or lack of it. Its reliability as a criterion of red cell production under varied circumstances needs further elucidation. Assuming reliability, it has marked advantages in that it shows an earlier response and greater magnitude of response than the other criteria available. Comparative studies of the various methods are in progress. The fact that one group using one method(15) finds plasma from bled-anemic animals totally inactive and others using different methods(5) find it sufficiently positive, illustrates the possible effect on conclusions that methodology may produce.

Stohlman *et al.*[§][¶] have reported erythropoietic stimulating effects of plasma from normal animals placed at various simulated altitudes, when given to sublethally irradiated recipients. 24-hour Fe^{59} uptake was the criterion used for presence or absence of response. We are unable to explain the discrepancy between their results and ours. The only significant differences in method were 1) their test animal was the sublethally irradiated rat whereas ours was either the normal or hypophysectomized rat. 2) Their altitude levels were simulated in decompression chambers whereas ours were accomplished using various mixtures of nitrogen and oxygen at ambient pressure. The degree of hypoxia was comparable. 3) Their animals were kept hypoxic for 24 hours whereas ours were kept so for 48 hours or longer.[||]

[§] Personal communication.

[¶] Reported at Sixth International Congress of Hematology.

Summary. Normal rats placed in a low oxygen atmosphere did not elaborate in their plasma a factor capable of stimulating increased incorporation of Fe^{59} into red cells of normal or hypophysectomized recipients. However, when their liver was acutely damaged with carbon tetrachloride and they were then placed in low oxygen atmosphere, significant amounts of such a factor appeared in their plasma. This is interpreted as further evidence that the liver, under ordinary circumstances, has a significant part in the mechanisms that control red cell production.

The authors express their thanks to André Bulba, Ruth Deckert, Gregory Makohon, Jane Shoemaker Edward Dywinski, and Helen Fox for technical assistance, and Dr. L. Simpson for pathological diagnoses of liver specimens. This investigation was supported in part by research grants from the Nat. Cancer Inst., USPHS and the Atomic Energy Com.

[||] Subsequent studies have shown 1) there is variation in susceptibility of groups of rats to the hepatotoxic effect of carbon tetrachloride. Only those with more severe damage show increased amounts of erythropoietic factor in their plasma at 48 hours, 2) increased levels of plasma erythropoietic factor have been found regularly in the plasma of hypoxic rats without liver damage at 24 hours. This has disappeared in every instance at the 48 hour interval.

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Uterotrophic Effect of Digoxin Administration in the Rat.[*] (23177)

EARL B. SCOTT AND WILLARD O. READ
Departments of Anatomy and Physiology and Pharmacology, School of Medicine,
State University of S. Dakota, Vermillion.

Le Winn(1) has described gynecomastia in men receiving prolonged digitalis medication for management of congestive heart failure. He suggested that digitalis steroids have an estrogen-like effect. A search of the literature has failed to reveal any description of estrogen-like stimulation in digitalized human or animal females. During the course of investigating the relationship between cardiotonic glycosides and protein metabolism, we observed that the uteri of mature ovariectomized female rats were trophically stimulated by the administration of digoxin. As a result, experiments were designed to further investigate this observation.

Material and method. Nineteen 68-day-old female Sprague-Dawley rats were bilaterally ovariectomized and divided into 4 groups following a 21-day post-operative period. The groups were treated as follows for

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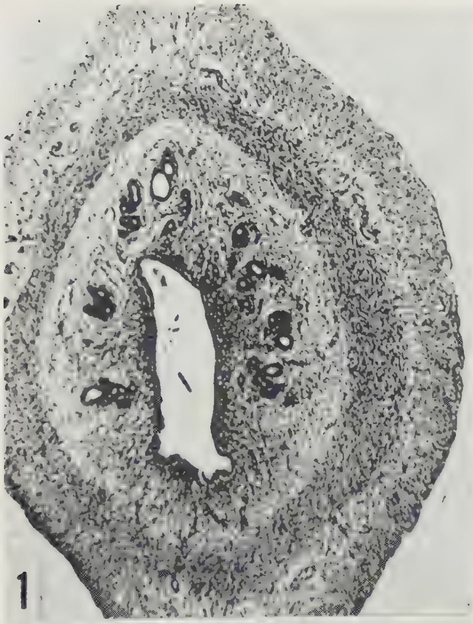
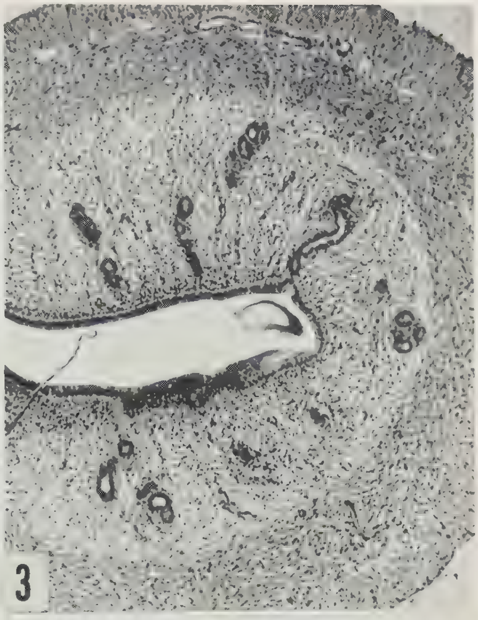
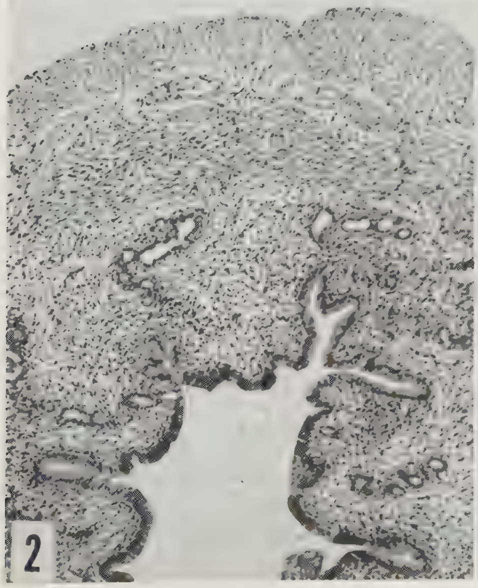
[*] This research was supported in part by Grant from Nat. Inst. of Arthritis and Metabolic Diseases, USPHS.







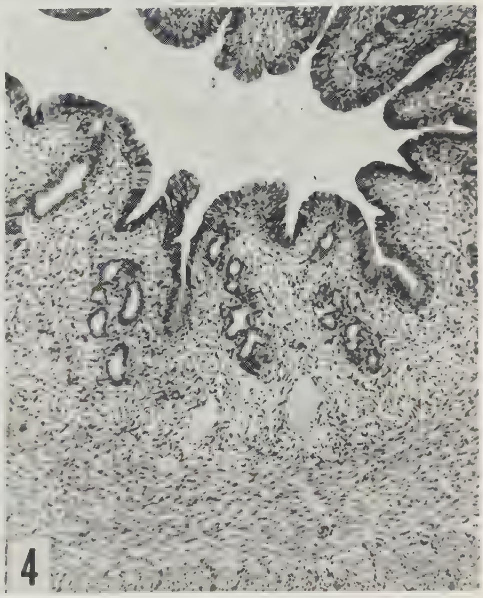


FIG. 1. Cross section of uterus of ovariectomized rat. ×35.

FIG. 2. Cross section of uterus of ovariectomized rat which received estrone. ×35.

FIG. 3. Cross section of uterus of ovariectomized rat which received digoxin. ×35.

FIG. 4. Cross section of uterus of ovariectomized rat which received both digoxin and estrone. ×35.

7 days: Group 1 (4 rats) with a mean weight of 239 g served as castrate controls; Group 2 (5 rats) with a mean weight of 262 g received a daily subcutaneous injection of digoxin[†] (2.0 mg/kg); Group 3 (5 rats) with

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[†] Digoxin was generously supplied by Burroughs Wellcome Co. who have recently adopted the trademark "Lanoxin" to facilitate distinction between digoxin and digitoxin.

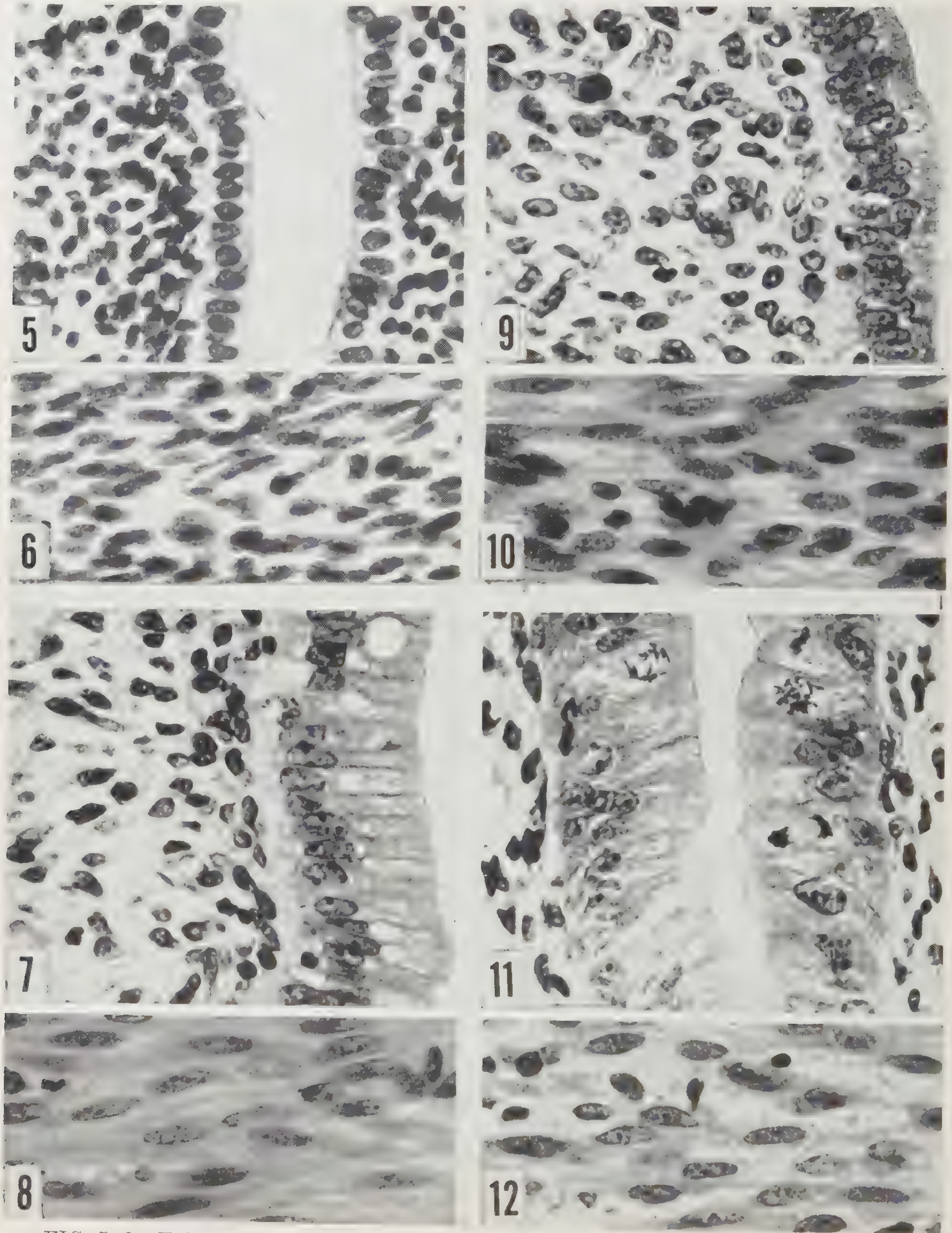

FIG. 5, 6. Epithelium, stroma and smooth muscle of ovariectomized rat uterus. ×344.

FIG. 7, 8. Epithelium, stroma and smooth muscle of estrone-treated ovariectomized rat uterus. ×344.

FIG. 9, 10. Epithelium, stroma and smooth muscle of digoxin-treated ovariectomized rat uterus. ×344.

FIG. 11, 12. Epithelium, stroma and smooth muscle of digoxin-estrone-treated ovariectomized rat uterus. ×344.

a mean weight of 244 g received 0.1 mg of estrone in oil subcutaneously on the first and fourth experimental days; Group 4 (5 rats) with a mean weight of 241 g received both digoxin and estrone in the above doses. Late in the 7th experimental day, all rats were sacrificed, their uteri removed, fixed in Bouin's solution, sectioned and stained with hematoxylin and eosin. A vaginal smear of each rat was made at this time.

Results. Castrate control. (Fig. 1, 5, 6). The uteri of these animals presented the typical well known reaction to bilateral ovariectomy. Grossly, the uteri were small and anemic. Histological sections showed a low epithelial lining, small endometrial glands, a compact stroma consisting of closely packed cells with intensely basophilic nuclei, and a myometrium composed of small muscle cells. The vaginal smears all showed the presence of the diestrus state.

Estrone-treated (Fig. 2, 7, 8). Estrone administration to castrate rats resulted in rapid recovery of their uteri. Grossly, these organs were large, hyperemic and moderately distended with fluid. In section, the endometrium was thrown up into longitudinal folds, the stroma was wide and edematous, the glands enlarged, and the myometrium well developed and repaired. The surface epithelium consisted of tall columnar cells with oval nuclei. Many of these cells showed vacuolar degeneration. Many mitoses were present and limited to the lining epithelium. The vaginal smears of these rats showed that they were in estrus by the presence only of cornified cells.

Digoxin-treated (Fig. 3, 9, 10). Digoxin administration to ovariectomized rats resulted in an unmistakable trophic response of the uteri. Grossly, these organs were large and hyperemic, but not distended with fluid. Microscopically, the endometrium was not folded, the lining being composed of a single layer of columnar epithelial cells, of which some portions were pseudostratified. These cells were considerably taller than those of the castrate controls but not heightened to the extent of those of the estrone-treated group. Their nuclei were long and oval occupying a central position in the cells. The stroma was edematous; the stromal nuclei were large and vesicular, each with a prominent nucleolus. The endometrial glands were enlarged. The smooth muscle cells of the muscularis were markedly increased in size and were completely recovered from the castrate condition. An occasional mitotic figure was observed in the lining epithelium and glands but none were seen in the stroma or muscle layers. The vaginal smears of 2 of these rats indicated that they were in late metestrus. These smears contained cornified cells, leucocytes and epithelial cells. Three rats had smears consisting only of epithelial cells, which is characteristic of proestrus.

Digoxin-estrone-treated (Fig. 4, 11, 12). The combined administration of both digoxin and estrone resulted in a response similar to, but much more intense than, that observed following estrone treatment alone. Grossly, these uteri were larger than any of the others, hyperemic, and enormously distended with fluid. Histologically, the endometrium was folded longitudinally and the lining epithelial cells were as tall as, and sometimes taller than, similar cells of the estrone-treated uteri. Their nuclei were large and located near the base of the cells. Some of these nuclei were karyorrhectic. The endometrial glands were enlarged. The stroma was edematous; the stromal nuclei were either vesicular or fusiform in character. The stromal blood vessels were considerably enlarged. The myometrium was recovered from the atrophic castrate condition. Mitoses were more numerous and prominent in the epithelium and glands of these uteri than in either the estrone-treated or digoxin-treated uteri. Mitoses were not observed in the stroma or myometrium. The vaginal smears of these rats were typically estrus, being composed of cornified cells only.

Discussion. The hypertrophic and hyperplastic responses of the castrate uterus to estrogen stimulation are well known. These are characterized by cell division; an edematous wide stroma; enlarged columnar epithelial cells; and a well developed, repaired myometrium. According to the criteria of Hooker(2), the most consistent and conspicuous change in the castrate mouse uterus treated with progesterone was vesiculation of the stromal nuclei and prominence of their nucleoli. He also noted the absence of interstitial edema, the lack of significant change in the epithelium and myometrium, and the high incidence of stromal mitoses.

Classification of the uterotrophic responses noted in the present study is difficult. The digoxin-treated uteri showed definite vesiculation of the stromal nuclei and prominent stromal nucleoli, but these were the only characteristics of progestational stimulation. On the other hand, the edematous stroma without mitoses, the hypertrophic surface epithelial cells and the repaired muscle cells may be used to classify the effect of digoxin as an estrogen-like response. The appearance of the vaginal smears of the digoxin-treated rats, some showing proestrus and some late metestrus, strongly suggests estrogenic stimulation, however small this stimulation may be. The magnitude of the changes evoked in uteri which received both digoxin and estrone suggests that the uterotrophic influence of digoxin may have been added to or superimposed upon the estrone effect.

The ultimate fate of digitalis glycosides is still disputed. Because of the structural similarity between cardiotonic glycosides and steroid hormones, one may speculate that a metabolite of digoxin, utilizing the cyclopentanoperhydrophenanthrene radical common to both groups of compounds, has estrogen-like qualities. Such a possibility as well as the roles of the pituitary, adrenals and liver are being investigated.

Summary. Digoxin administration to mature ovariectomized rats resulted in uterotrophic response evidenced by heightening of epithelium and enlargement of epithelial nuclei, edema of the stroma with vesiculation of stromal nuclei and prominence of stromal nucleoli, and recovery of the myometrium. The response of castrate uteri to estrone was intensified by the concurrent administration of digoxin.

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Production of Gastric Ulcers in Dogs on Protein Depletion Regime.* (23178)

P. F. HAHN, PERCY BAUGH, AND DOUGLAS L. FOSTER

Cancer Research Laboratories, Meharry Medical College, Nashville, Tenn.

In higher mammals production of hemoglobin takes precedence over formation of most body proteins. Advantage was taken of that fact in 1939 when we were able to demonstrate that in the dog, hemoglobin production could be limited by protein intake(1). By "forced hemoglobin production" in dogs concomitantly maintained on a low protein intake circulating and reserve proteins were rapidly depleted. In recent studies in which a similar technic of protein reduction was used in studies of anaphylaxis(2) and homo-transplants[†] we have been impressed with the high incidence of gastric ulcer and particularly where a perforation was the immediate cause of death. These lesions varied quite considerably as to severity and location. In some instances there were patchy and in others quite extensive denudement of the gastric mucosa. In a few, deep indurating ulcers involved not only the mucosa but the muscularis. Very frequently complete perforation of the stomach occurred with appearance of "punched out" lesions frequently seen at human autopsies. Some of these changes can be seen in Fig. 1, 2 and 3. Most frequently the lesion was seen on the greater curvature or above the pyloric sphincter and uncommonly in the duodenum.

Methods. The procedure can be described briefly as follows: The dogs were bled ¼ of estimated circulating blood volume (72 ml/kg) on each of 3 successive days. The same

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* Carried out under contract with Division of Biology and Medicine, U. S. Atomic Energy Com.

[†] In preparation.

TABLE I. Low Protein Diet Used with Forced Hemoglobin Production.

	g	Prot.	Fat (g)	CHO	Total cals.
Bananas	300	3.6	.6	69	300
Salmon	100	20.6	9.6	0	168
Cod liver oil	40		40		360
Karo syrup	120			120	480
Wesson salts	1				
	560	24	50	190	1308
		7.4% prot. cal.	34% fat cal.	58% CHO cal.	

Fed 50 g/kg = 117 cal./kg.

amount of blood was removed on the 5th day and this usually resulted in establishment of a temporary anemic state at about ½ normal hematocrit value. Phlebotomy was then carried out at such intervals as to maintain the hematocrit at approximately this level. From this time iron as saccharated oxide ("Proferrin" was administered at time of each bleeding in amounts of approximately 1 mg for each ml of red cells removed. Normally this would result in maintenance of iron balance and since under this procedure, the erythrocytes become hypochromic, one was giving essentially an excess of iron over and above that removed. From the time of establishment of the anemic state, the dogs were maintained on a diet low in proteins as well as other dietary essentials such as certain vitamin fractions. Its composition is shown in Table I.

Results. A group of 6 litter-mate dogs was being depleted of proteins by the method described above. Two of these animals died of perforating ulcers 6 and 7 months respectively after institution of the diet, bleeding and iron injections. This called our attention to the incidence of such changes and we realized that in the past we might have missed at autopsy a number of instances in which nonperforating ulcers had existed in the stomach or duodenum. Therefore, in the present communication we shall be concerned mainly with the next 15 animals which were placed on this regime and examined carefully at autopsy for gastric and duodenal lesions.

Two litter-mate animals, 55-7 and 55-8, were treated by the same procedure and died in 3.5 months of perforating ulcers. The first had an ulcer of the lower greater curvature which had perforated and also other ulcers involving the gastric and duodenal mucosa. The second had perforating pyloric and duodenal ulcers. The next group of 4 litter-mate dogs, 55-53 through 55-56, on the same regime with no other treatment survived 2, 3, 3 and 3.5 months respectively. Three out of 4 of these animals showed lesions of the mouth and fore and hind legs. Two had no ulcerative changes in the gastro-intestinal tract. On the other hand, 2 of them had perforating pyloric ulcers and 1 of these in addition had a perforating duodenal ulcer. The other with the perforating gastric ulcer showed a hemorrhagic ulcer of the greater curvature. The next 3 animals were littermates 55-82 through 55-84. These animals were on the same regime as the other dogs except that 2 of them received daily supplements of vitamins as follows: Thiamine = 50 mg; riboflavin = 5 mg; nicotinamide = 50 mg; ascorbic acid = 12 mg and Brewers yeast = 1.5 tablets daily. The third animal received no vitamins. The latter animal survived 5 months and cause of death was unknown. At autopsy the gastric wall showed internal hemorrhages and the usual fatty liver was present. One of the vitamin treated animals was sacrificed at this time for comparative purposes and showed a normal stomach. The liver showed grossly very slight fatty infiltration. The second animal receiving vitamins died 8 months after being placed on this regime and showed internal hemorrhage of the gastric wall.

The next 7 dogs were litter-mate animals, 55-43 through 55-49, of which one died of extraneous causes before the regime began. Three months after beginning the low protein-forced hemoglobin production regime, 3 of the animals were given a single intravenous injection of radioactive colloidal gold of 30 mc. Following depletion of their circulating protein they were all used as candidates for skin homografts following which they were later studied for tuberculin sensitivity 4 weeks following subcutaneous administration of bacto-adjuvant. It is interesting to note that 5 out of 6 of the animals survived for 5

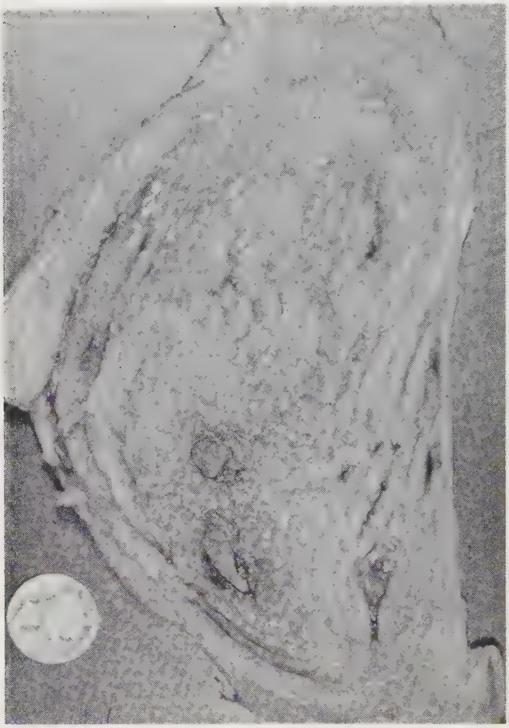

FIG. 1. Dog 55-48: Internal wall of stomach showing 2 indurating ulcers on curvature.

months all dying spontaneously within a period of 1 week. The last animal survived 6½ months. Four of the 6 animals showed absence of mucosa in the greater curvature. The 2 others showed deep indurating ulcers of the pyloric mucosa and duodenum. Two of the animals had, in addition, perforating ulcers above the pyloric sphincter.

The animals on this regime lost a considerable amount of weight and, indeed, this was the most striking feature as far as clinical signs were concerned. Elsewhere(2) we have shown that the electrophoretic patterns of dogs on such a regime undergo a considerable change as far as distribution of the plasma protein constituents although the total plasma proteins are not markedly altered.

Aside from the high incidence of peptic ulcer in these animals several other points should be mentioned briefly. In every case there was a very considerable amount of deposition of subcutaneous fat varying from 1.5 to 3 cm in thickness. This was such as to preclude the successful use of skin homografts. This factor will be described in detail elsewhere. This lipid deposition, it should be noted, took place in spite of the uniform degree of emaciation in these animals. It was very nearly liquid in appearance suggesting a high degree of unsaturation. In all animals there was at autopsy gross evidence of extensive fatty infiltration of the liver although in the 2 vitamin treated animals this seemed to be somewhat less than in the others. In most of the animals there also developed rather deep ulcerative lesions usually involving the mouth and quite characteristically also appearing at the ankle joints. We had noted these lesions in our earlier work involving the use of forced hemoglobin production in protein depletion(1) and found that such lesions very promptly disappeared within a few days following the administration of whole liver.

Of the 15 dogs consecutively used the time from institution of the low protein-forced hemoglobin regime to death averaged 4.5 months.

Discussion. Li and Freeman(3) reported 47% incidence of peptic ulcers in dogs maintained on diets which were free from protein except for that contained in yeast which made

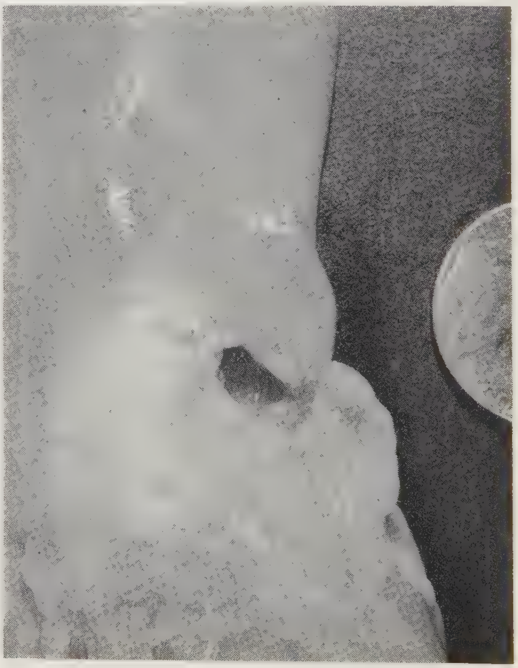

FIG. 2. Dog 55-7: Exterior wall of lower portion of stomach and duodenum showing perforating ulcer.

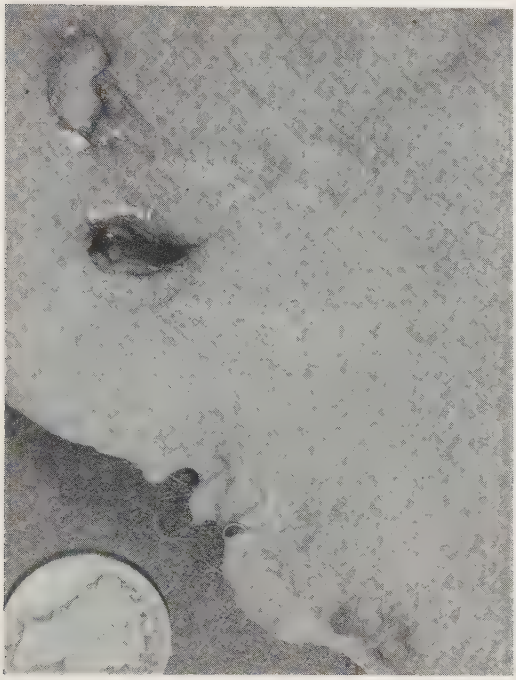

FIG. 3. Dog 55-48: External wall of stomach showing one indurating and one perforating ulcer near pyloric area.

up 5% of each diet. Twelve weeks on such a regime seemed to be the minimum of time required for development of any peptic ulcer. Of 32 protein deficient dogs studied 15 had solitary peptic ulcers, 13 of which animals had ulceration on the pyloric side of the sphincter. Fouts(4) fed dogs a low protein (casein) diet supplemented with thiamine, nicotinic acid, riboflavin, pyridoxine and pantothenic acid over extended periods of time. They lived on an average of 175 days developing anorexia, weight loss, anemia, skin ulcers, peptic ulcers and fatty cirrhotic livers. Weech and Paige(5) maintaining their dogs in the study of nutritional edema on a diet consisting of carrots, rice, lard, cod liver oil and sugar and salt mixture found a high incidence of peptic ulcers of the stomach and duodenum. Among 22 animals receiving the diet for an average of 90 days 36% exhibited true peptic ulcers at autopsy. Of the remaining 14, 23% showed erosions of gastric or duodenal epithelium without true ulcer formation. The incidence of ulcers could not be correlated with duration of the diet, degree of emaciation as shown by weight loss or degree of anemia which was established. There seemed to be the possibility of a correlation between serum albumin levels with development of the ulcers. Other investigators have described occurrence of gastro-intestinal ulcers in rats and mice maintained on various protein deficient diets.

We do not feel that induction of skin grafts or sensitization to bacto-adjuvant with subsequent tuberculin test were likely to have affected the induction of the ulcerous lesions. The incidence of overall lesions is higher in that group than in the group of dogs as a whole but the number involved is too small to permit evaluation. The closeness of time of death in the siblings of this group however is very striking as mentioned above.

Simple iron-deficiency anemia maintained in dogs by phlebotomy while on normal or low-iron diets over many years does not result in gastric lesions in the experience of one of the authors (PFH).

On the basis of the findings we are not prepared to speculate on the relationship of lowered and altered distribution of circulating proteins or depletion of body protein stores on the induction of ulcers.

Summary. Dogs maintained in the anemic state by phlebotomy with intravenous injection of amounts of iron equivalent to that removed in hemoglobin and concomitantly fed a low protein diet, are quite rapidly depleted of their protein reserves and the circulating plasma proteins are markedly altered. Weight loss is invariably considerable. In these animals the incidence of peptic ulcer is extremely high with a large percentage developing perforating ulcers causing death.

The authors are indebted to Primus Mootry for able technical assistance.

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Increase in Red Cell Volume in Response to Long Exposure to Cold.* (23179)

J. F. Garcia, R. W. Belknap, and S. F. Cook
The Donner Laboratory and the Department of Physiology, University of California, Berkeley.

Although blood and plasma volume changes have been given some attention in studies involving exposing animals to cold environments, very little mention has been made regarding the red cell picture. Bass and Henschel(1) have recently reviewed the subject of volume changes in cold environments. Baxett *et al.*(2) report a decrease in blood volume and total circulating hemoglobin in humans after prolonged exposure to cold. However, Brown *et al.*(3) observed that the blood volume of the Eskimo was high and that it fell in the summer months and suggested that this was associated with cold acclimatization. In the rat, Deb and Hart(4) observed increased blood and plasma volume after 5 weeks exposure to an environment of 6°C.

Since it is well substantiated that oxygen consumption is increased in rats exposed to a cold environment(5,6), and since one of the functions of hemoglobin is the transport of oxygen, it would seem pertinent that the state of erythropoiesis should be reinvestigated. Use of labelled red cell dilution technic was employed here to see if there is any net gain in total red cell volume in rats exposed to a cold environment.

Methods. The rats were males of the Long-Evans strain, fed a complete laboratory diet.† Thirty-six rats, approximately 170 days of age, were divided into 2 groups so that the average weight of each group was the same at the beginning of the experiment. The control group was maintained at room temperature and the cold-exposed group was kept at 3°C ± 1°C. At the end of 30 days, blood volumes were determined by use of Fe^{59} labelled red cell dilution technic(7). The labelled red cells were obtained by cardiac puncture from a donor animal previously injected with Fe^{59}. *Blood volume determinations* were done under ether anesthesia. Injections of tagged blood were made through the saphenous vein which was exposed by a small skin incision. A specially calibrated 0.25 ml tuberculin syringe was used so that a constant volume of donor blood was injected into each animal. Using this syringe, the same volume of donor blood was put into a 10 ml volumetric flask, which when diluted and an aliquot pipetted and counted, gave a measure of the amount of Fe^{59} activity which was injected into each animal. After injection, 6 minutes were allowed for the donor blood to mix, at the end of which time the abdomen was opened, and as much blood as would flow freely was drawn from the vena cava into a heparinized syringe. A portion of this blood was used for an hematocrit and hemoglobin determination and an aliquot was counted directly in a scintillation counter adapted for vial counting(8). The amount of radioactivity injected was divided by the amount of radioactivity/unit of recipient blood to obtain "blood volume." The total "blood volume" multiplied by the hematocrit gave total red cell volume. The total circulating hemoglobin was obtained by multiplying total blood volume by the hemoglobin concentration.

Results. A summary of the data is presented in Table I. The differences between normal and exposed rats observed in the hematocrits and hemoglobin concentrations were not statistically significant. However, total red cell volume and total circulating hemoglobin of the cold exposed rats increased by 12.9 and 10.9% respectively. These values are significant with a "P" value of less than 0.001.

Stullken and Hiestand(9) observed no change in either hemoglobin concentration or erythrocyte number in mice after a 5 day ex-

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* This work was supported in part by Atomic Energy Commission and University of California.

† The diet was 'Simonsen Green Diet' obtained from Simonsen Laboratories, Gilroy, Calif.

TABLE I. Summary of Hematological Data of Rats Exposed to Cold Environment for 30 Days; 18 Rats/Series.

	Body wt, g		Hemato-crit, %	Hemoglobin conc., g/100 ml blood	Total blood vol, ml	Total red cell vol			Total circulating hemoglobin		
	Initial	Final				ml	% change	P*	g	% change	P
Controls	400	428	45.5	13.2	20.22	9.20	12.9	.001	2.67	10.9	.001
Cold exposed 30-days	400	413	47.2	13.4	22.01	10.39			2.96		

* Fisher's "t" test(10).

posure to cold. However, they suggest that possibly 5 days is not a long enough time in which to expect acclimatization to appear. The present study would also suggest that concentration studies alone are perhaps not as conclusive as the observation of a net increase in red cell volume in response to a particular stimulation.

That an increase in red cell volume is observed in animals exposed to a cold environment is not altogether unexpected when one considers that it is accompanied by a physiologically increased requirement of oxygen. Such a stimulation of red cell production, possibly secondary to an increased oxygen consumption, is interesting since in many different endocrine states variations in the red cell picture are seen accompanied by variations in oxygen consumption.

Summary. An increase in total red cell volume and total circulating hemoglobin was observed in rats exposed to a cold environment for a period of 30 days.

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Bromsulphalein Dye Retention Test in Toxicological Investigations. (23180)

RODERICK A. E. THOMSON, DAVID B. MCKAIG,* AND SOL M. MICHAELSON†
(Introduced by T. R. Noonan)
Eaton Laboratories, Norwich, N. Y.

Routine toxicologic investigations demand evaluation of liver function in the experimental animal as an indication of possible hepatotoxicity. The method employed should give results which can be read quantitatively. This report describes a procedure by which concentrations of the bromsulphalein dye in the blood are obtained which can easily be determined by standard electrophotometric methods. Moses *et al.*(1) have reported that bromsulphalein at doses of 5, 10 and 20 mg/kg intravenously in dogs yielded reproducible and quantitatively similar curves. Gornall *et al.*(2) showed that a 10

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* Present address: Charleston, O.

† Present address: University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

TABLE I. 15 Minute B.S.P. Retention Values for 5 Rabbits Fed CCl_4.

% bromsulphalein retention					
Control	After 2 doses CCl_4	After 4 doses CCl_4	7 days following 4th dose	14 days following 4th dose	Liver damage
4.6	30	64	8.5	6.4	Mild
4.0	9.7	36*	—	7.0	”
6.5	Dead	—No autopsy performed—			
4.5	”	—Autopsy	”	—	Very marked
5.7	45.6	92	Sacrificed, autopsy performed		Marked

* Retention at 18 min.

mg/kg dose administered intravenously gave more satisfactory results than the 5 mg/kg dose. Smith *et al.*(3) showed that in many instances 15 minutes after administering the dye none was found in the blood, indicating rapid removal of the dye from the blood stream.

To evaluate possible hepatotoxicity of agents with potential chemotherapeutic value, an indication of altered liver function should be of diagnostic significance. The bromsulphalein dye retention test serves as a useful method for evaluation of liver function.

Materials and methods. Male and female adult albino rabbits were used in these experiments. Bromsulphalein dye was given intravenously at a dose of 10 mg/kg. 20 mg of dye per 100 ml of blood was considered 100% retention(2).

Results. Control bromsulphalein retention value at 15 minutes after injection of the dye was determined on 63 rabbits, with a mean value of 3.8% ± 1.6.[‡] Five of the rabbits were given 5 mg/kg of a 10% (v/v) solution of CCl_4 in peanut oil by gavage, twice weekly, to produce liver impairment(4). Two of the 5 rabbits died after receiving 2 doses of the CCl_4, the other 3 received a total of 4 doses (Table I). Retention values at 5, 10, 15, 30, and 60 minutes were obtained on 2 of the 5 rabbits to establish control curves (Table II and Fig. 1).

After 2 and 4 doses of CCl_4, 15 minute retention values were determined on the 3 remaining rabbits (Table I). Simultaneously, retention values at 5, 10, 15, 30, and 60 minutes were obtained on rabbit 142, on which a control curve had already been established (Table II). All tests were done approximately 24 hours after the CCl_4 was given. The rabbits were fasted overnight prior to the determinations.

Autopsy was performed on rabbit 140, which died after receiving 2 doses of CCl_4, and on rabbit 142, which was sacrificed after receiving the 4 doses. Gross examination revealed marked liver damage in rabbits 140, and 142. The findings were corroborated on examining tissue sections of the liver. Liver

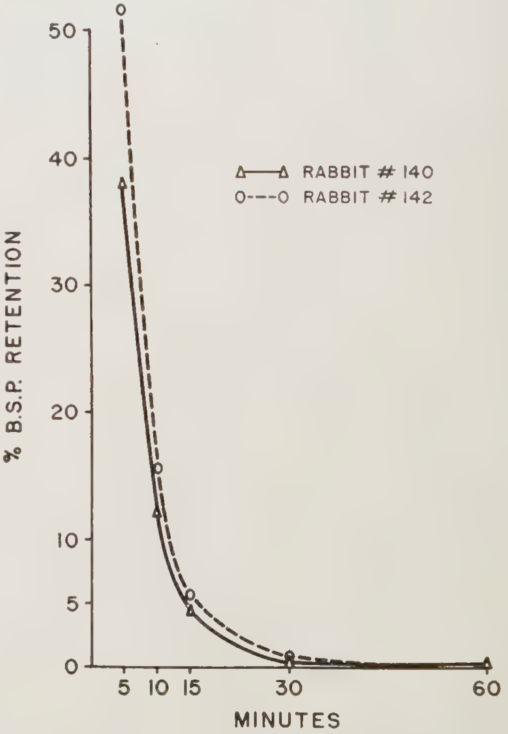

FIG. 1. Control B.S.P. retention values for rabbits No. 140 and 142.

[‡] Stand. dev.

TABLE II. Bromsulphalein Value at Selected Time Intervals for Rabbits Fed CCl_4.

Specimen	% bromsulphalein retention 5 min.	10 min.	15 min.	30 min.	60 min.
Control	51.2	15.7	5.7	.7	0
After 2 doses	91.2	69.8	45.6	33.8	5.2
After 4 doses		110.6	92	50	22
Control	38	12.2	4.5	.5	.3

sections from rabbit 140 revealed greater liver damage than that of rabbit 142. Liver sections from rabbits 139 and 148 which were killed 14 days following the fourth dose of CCl_4 revealed milder liver damage (Table I). No tissue was obtained from rabbit 138 as death occurred on a weekend and the rabbit was disposed of by error.

Summary. 1) Fifteen minutes after administration of bromsulphalein at a dose of 10 mg/kg i.v. in rabbits, dye was present in the blood and a satisfactory determination of the dye concentration was possible. 2) Liver damage was reflected by an increased retention of bromsulphalein 15 minutes after injection of the dye.

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Excretion of Urinary "Epinephrines" in Psychiatric Disorders.* (23181)

HIRSH SULKOWITCH, GEORGE M. PERRIN† AND MARK D. ALTSCHULE
Laboratory of Clinical Physiology, McLean Hospital, Waverley, Lahey Foundation, Boston, and Department of Medicine, Harvard Medical School, Boston, Mass.

Physiologic changes which resemble those following injection of epinephrine and norepinephrine frequently occur in response to many types of noxious stimuli. The recent introduction of bio-assay and chemical procedures for estimation of epinephrine has made it possible to confirm physiologic evidence of increased sympathetic medullary activity under these circumstances. Since fear or anxiety is one of the most prominent symptoms in both neurotic and psychotic patients, such a study is of particular interest in these individuals. The purpose of this report is to present preliminary observations on excretion of urinary epinephrine in this group.

Materials and methods. Seventeen pyschiatric patients, none of whom was receiving a course of tranquillizing drugs, insulin treatment or electric shock therapy, were studied on one or more occasions. Diagnoses of the nature of the disorders were made by the psychiatric staff of McLean Hospital. In some cases, the illness was acute, while in others it was chronic. Some, but not all, patients complained of severe anxiety and often showed physiological evidence of increased epinephrine activity such as clammy hands and tachycardia. Their blood pressures, however, were within normal limits. The method used in determination of the urinary "epinephrines"‡ has been described elsewhere(1). Minor refinements in the chromatographic technics were made during the course of the investigation. In a control series comprising 45 patients, values ranged from 0 to 59.5 μg per 24 hours; average value was 20.0 and standard error was 2.9. Values in patients

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* Aided by grants from Donner Foundation and Lahey Foundation.

† Public Health Service Research Fellow of Nat. Heart Inst.

‡ "Epinephrines", as used in this paper, includes all compounds detected by the method employed. These substances include epinephrine, norepinephrine, certain other alpha-hydroxy catechol amines, trihydroxindoles, and 3,5 hydroxy-6-oxy indoles.

TABLE I. Value of Urinary ''Epinephrines'' in Psychiatric Patients.

Case			Urinary ''epinephrines,'' μg/24 hr
	Age	Diagnosis	
45 controls			0-59.5, Avg 20 ± 2.9*
♀	40	Psychotic disorder, psychotic depressive reaction	773
♂	26	Schizophrenic reaction, catatonic type	0
''	30	*Idem*	631 184 639 728 203 360
♀	26	Schizophrenic reaction, paranoid type	577 270
''	42	*Idem*	730
♂	38	Schizophrenic reaction, schizo-affective type	800
♀	45	*Idem*	353 339
''	46	''	710
♂	21	Psychoneurotic disorder, anxiety reaction	660
♀	36	*Idem*	364
''	34	Psychoneurotic disorder, dissociative reaction	1184 1055
''	36	Psychoneurotic disorder, obsessive compulsive reaction	133
''	24	Psychoneurotic disorder, depressive reaction	237
''	''	*Idem*	233
''	51	''	119
♂	16	Personality disorder, passive aggressive disturbance	34
''	14	Transient situational personality disturbance, adjustment reaction of childhood, conduct disturbance, destructiveness	3

* S.E.

with pheochromocytomas are usually from 200 to 3000 μg, but on rare occasions normal findings may be present in patients with these tumors.

Results. The urinary "epinephrines" of most of the patients were from 200 to 500 μg per day (Table I), values well in excess of those found in normal individuals. However, three patients, one recently recovered from catatonic stupor, one with a personality disorder and the third an adjustment problem, showed normal values. The 2 latter subjects were the youngest in the series and could be more properly classified as suffering from personality disorders rather than from psychoneurosis or psychosis. The highest readings for neurotic patients and schizophrenic patients were 1184 and 800 μg respectively.

Comment. The literature on urinary epinephrine and norepinephrine concentration in both normal subjects under stress and in psychiatric patients is sparse(2-5). Some normal and some elevated values have been found in neurotic and psychotic patients. Increased urinary outputs have been reported on occasions in normal individuals subjected to stress.

For almost a half century, substantial physiological evidence(6,7) has accumulated which suggests increased secretion of epinephrine or norepinephrine in response to strong emotional stimuli. Although these data are based on acute experiments, there is little reason to doubt that the chronic anxiety of neurotic and psychotic patients might eventuate in such increased activity of the adrenal medulla and sympathetic nervous system.

The problem arose of whether urinary "epinephrines" could be correlated with subjective anxiety or with physiologic evidence of increased sympathetic activity. Since all current methods of measuring both anxiety and the status of the autonomic nervous system are crude, a systematic study was impossible. However, neurotic patients with high values seemed in general to be more anxious and more subject to excess perspiration and palpitations. Such a relation was not so evident in the psychotic group; for example, one patient showed normal values within 2 weeks after an episode of catatonic stupor. Although none of the subjects of Table I were hypertensive, 2 patients studied at another hospital, who exhibited both anxiety and hypertension while acutely ill, showed elevated urinary "epinephrines" (375 and 670 μg) during this period. With the diminution of anxiety, these values fell significantly to 107 and 92 μg respectively, and blood pressure readings fell to normal levels.

The finding of increased "epinephrines" in neurotic and psychotic patients raises, of course, the question why physiological and biochemical abnormalities are observed less frequently in this group than in patients with pheochromocytomas. This question cannot be answered fully; however, one factor might be the increased rate of oxidation of epinephrine commonly found in schizophrenia(8).

Summary. Increased 24 hr excretion of urinary epinephrines was found in 14 of 17 patients with various psychiatric disorders. These increased outputs varied from about 200 to 500 μg/day; the largest was 1184. Such values are within the range of those found cases of pheochromocytoma and emphasize the fact that increased epinephrine excretion is not pathognomonic of adrenal medullary and chromaffin chain tumors.

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Effects of Aminopterin and Estrogen on Phosphoprotein Phosphatase of Rat Uterus.* (23182)

JAMES S. DAVIS (Introduced by Roland H. Alden)
Division of Anatomy, University of Tennessee Medical Units, Memphis.

In a previous investigation of the effects of estrogen and 4-aminopteroylglutamic acid (Aminopterin) on phosphate metabolism of rat uterus(1), it was observed that Aminopterin caused an almost complete inhibition of synthesis of phosphoprotein in response to estrogen. Aminopterin also inhibited uterine growth in response to estrogen which suggested that synthesis of phosphoprotein, might be a significant factor in growth of this organ. Therefore, it was felt that a study of the enzyme concerned in metabolism of uterine phosphoprotein would be of interest.

Methods. 45 female rats of the Holtzman strain, weighing 45-55 g, were used. The animals were 22 days old when spayed and were placed on experiment 1-2 days after spaying. They were grouped according to treatment as follows: Group AE contained 22 animals, receiving 10 μg Aminopterin/day for 4 days intraperitoneally and 0.1 μg 17-β-estradiol/day for 3 days subcutaneously. Estrogen was given simultaneously with the last 3 Aminopterin injections. Group E contained 6 animals which received estrogen injections only and Group C contained 17 animals serving as untreated controls. At necropsy uteri were removed, stripped free of mesometrium, weighed, pooled as described in Table I, and 10% water homogenates prepared. Assay procedure for phosphoprotein phosphatase activity was that described by Norberg(2) except that whole homogenates rather than extracts were used. The substrate was commercial casein dissolved in dilute NaOH solution and precipitated with HCl 3 times, then stored in frozen state. This procedure was necessary to prevent precipitation of proteoses after addition of molybdate in phosphate determinations. Inorganic phosphorus was determined according to Lepage(3).

Results. Activity of phosphoprotein phosphatase in the different groups (Table I) cannot be correlated with observations on phosphoprotein fraction of uterus studied under identical experimental conditions, as described previously(1). Values for this

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TABLE I. Phosphoprotein Phosphatase Activity of Rat Uterus.[*]

Group	Body wt change, g[†]	Uterine wt, mg[‡]	No. determinations[§]	Units, µM P/min./g [‡][‖]	Total × 10^{-3} [¶]
C	+16	25.6 ± 1.061	3	1.636 ± .075	41.85
AE	−11	29.8 ± 2.162 (>.05)	4	2.297 ± .008 (<.01)	68.45
E	+15	86.7 ± 2.173 (<.01)	3	1.961 ± .151 (>.05)	170.0

[*] Mean values and stand. error.

[†] Gain or loss in body wt during 4-day injection period.

[‡] Significant difference between means of exp. and control groups indicated by P = .05 or less, shown in parentheses.

[§] 2-7 uteri pooled for each determination.

[‖] µmoles phosphorus released/min./g fresh tissue.

[¶] Units × avg uterine wt × 10^{-3}.

fraction (taken from previous publication) have been included in Fig. 1 to facilitate comparison with enzyme activity. Total phosphoprotein/uterus is used as index of phosphoprotein synthesis and incorporation of radioactive phosphorus by this fraction is included as a relative index of turnover. P^{32} uptake was calculated as counts/min/µg P divided by injected dose.

In response to estrogen alone (Group E) phosphatase activity/g fresh tissue did not increase significantly over the control value but total activity/uterus increased as a consequence of 240% increase in uterine weight. Synthesis of phosphoprotein, however, increased 810% while P^{32} incorporation decreased 55%. In Group AE, where Aminopterin completely inhibited uterine growth in response to estrogen, phosphatase activity increased significantly, but synthesis of phosphoprotein was not significantly greater than in controls, and turnover increased 300%. It appears, then, that phosphoprotein synthesis and turnover are not directly related to phosphatase activity and phosphoprotein turnover is inversely related to synthesis of phosphoprotein and uterine growth.

Discussion. A similar discrepancy between activity of phosphoprotein phosphatase and synthesis of phosphoprotein in a growing organ was observed by Norberg(4), who studied regeneration of rat liver after partial hepatectomy. Total enzyme/liver increased at the same rate as liver mass but enzyme concentration did not change appreciably, as observed for uterus in Group E. Total phosphoprotein likewise increased at the same rate as liver mass except on the 3rd and 10th days of regeneration when phosphoprotein increase was 50% greater than the increase in liver mass. In this respect the 3rd and 10th days of regeneration are similar to the finding for Group E uteri. Also, Johnson and Albert (5), who studied phosphoproteins of various rat organs, noted an inverse relation between phosphoprotein content and turnover rate, and a lack of correlation between enzyme activity and turnover.

At present only a theoretical explanation can be offered for the results presented here. It is possible, if not probable, that phosphoprotein phosphatase functions only as a

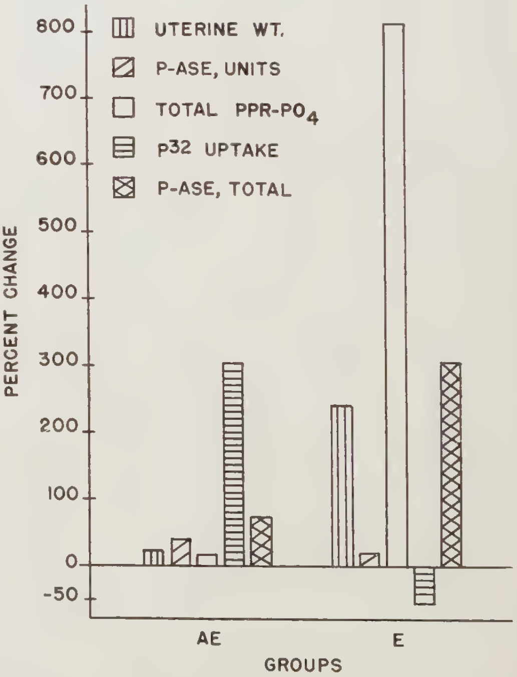

FIG. 1. Experimental results calculated as % of control value. Values for phosphoprotein fraction taken from Davis, Meyer, and McShan(1).

catabolic enzyme that is controlled by a specific protein inhibitor, similar to the inhibitor described for deoxyribonuclease(6). Such an inhibitor would not necessarily be apparent in the assay procedure for enzyme activity. In Group AE Aminopterin apparently interferes with synthesis of inhibitor, which is consistent with ability of Aminopterin to inhibit incorporaton of glycine and serine carbon into proteins(1). Estrogen in Group AE stimulates synthesis of phosphoprotein but hydrolysis proceeds at an equal rate, thus preventing a net increase in phosphoprotein and accounting for the very high turnover of phosphorus. In Group E production of inhibitor would be unimpeded, preventing the high turnover rate and resulting in increased accumulation of phosphoprotein.

Harris(7) observed that some controlling factor must exist for phosphoprotein phosphatase of frog eggs but he believed that pH of the yolk was that factor. Johnson and Albert(5) suggested that a specific inhibitor might exist in mammalian tissues, but the hypothesis obviously requires direct experimental support, particularly in view of Feinstein's unsuccessful attempt to demonstrate such an inhibitor in rat intestine(8).

Summary. Total phosphoprotein phosphatase activity of rat uterus increases in response to estrogen as uterine weight increases, but activity/g fresh tissue does not change appreciably. Aminopterin administered simultaneously with estrogen inhibits the increase in total activity/uterus as it inhibits uterine weight increase. The changes in enzyme activity could not be correlated with changes in uterine phosphoprotein and the possible existence of a phosphatase inhibitor was discussed.

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Influence of Water-Treatment on Nutritional Value of Barley.* (23183)

RAMON E. FRY, JOHN B. ALLRED, LEO S. JENSEN AND JAMES MCGINNIS
Department of Poultry Science, State College of Washington, Pullman.

Fry *et al.*(1) showed that feeding value of barley for chicks was not improved greatly by removal of the fibrous hull through pearling. Results on metabolizable energy content analysis (with chicks) of barley and pearled barley obtained on material sent from our laboratory showed that pearled barley contained *less* metabolizable energy than regular barley.† These findings suggested that the carbohydrate in barley, aside from crude fiber, is much less available than similar carbohydrates in corn. Studies were undertaken to determine whether the nutritional value of barley could be improved by different treatments. The results obtained in 2 of these experiments are here presented.

Procedure. As the basis of a working hypothesis, it was assumed that the lower nutritional value of barley could be due to a number of reasons such as: (1) Presence of an inhibiting substance or substances; (2) existence of structural linkages in the carbohydrates of barley that are not attacked by enzymes endogenous to the chick; or (3) outright deficiency in carbohydrate content of

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† The authors are indebted to Dr. F. W. Hill of Cornell University for determination of the metabolizable energy values.

barley. The third possibility did not seem logical since the proximate analysis of barley or pearled barley indicates that this grain has as much or more carbohydrate than corn. It was decided, therefore, to select a treatment which might destroy or counteract inhibitors or alter the structure of the carbohydrates. McGinnis and Polis(2) showed that simple water treatment of linseed oil meal markedly improved nutritional value of this protein supplement. This improvement was later shown by Kratzer(3) to be through the inactivation of a pyridoxine antagonist. A water treatment of pearled barley was, therefore, undertaken. It was also felt that such a treatment might alter the carbohydrates in barley by permitting action of enzymes contained in the grain. Coarsely ground pearled barley was mixed with an equal weight of tap water (40°C) and allowed to stand at room temperature (21°C) approximately 8 hours. By the end of this time all of the water was absorbed by the grain. The wet barley was then spread on trays to a depth of one to 2 inches and placed in a forced draft electric oven at 70°C for approximately 15 hours, or until dry. It was then ground in a hammer mill through a ⅛ inch screen. No attempt was made to prevent or retard microbial action during the above treatment. Similar procedures were used in conducting the 2 chick experiments. Three replicate groups of 10 New Hampshire chicks each were fed each diet in Exp. 1, with the exception of the water treated pearled barley diet in which only 2 replicates were used. In Exp. 2, 3 replicate groups of 9 chicks each were given the experimental diets. The chicks were maintained from day-old to 4 weeks in electrically heated batteries with wire screen floors. Feed and water were supplied *ad libitum*. Composition of the diets for each study was the same except for the grain component. The percentage composition of diets was as follows: Grain (corn or pearled barley), 64.2; soybean oil meal (50% protein), 24.3; herring fish meal, 5.0; dehydrated alfalfa, 2.5; bone meal, 2.5; limestone, 0.5; salt, 0.3; premix, 0.7. The premix supplied the following/pound of diet: vit. A, 1200 I.U.; vit. D, 200 I.U.; vit. E, 5 I.U.; riboflavin, 2 mg; Ca Pantothenate, 4 mg; niacin, 7 mg; choline Cl, 400 mg; penicillin, 2.5 mg; methionine, 227 mg; $MnSO_4$, 60 mg; Butylated hydroxy toluene, 57 mg.

TABLE I. Effect of Water Treating Pearled Barley on Chick Growth and Feed Efficiency.

Grain in diet	Avg wt at 4 wk		Feed/gain	
	By replicate	By treatment	By replicate	By treatment
Exp. 1				
Corn	365		1.87	
	364	363*	1.95	1.93*
	361		1.97	
Pearled barley	299		2.19	
	309	306	2.25	2.31
	310		2.50	
Water-treated	406		1.72	
pearled barley	431	420*	1.71	1.72*†
Exp. 2				
Corn	382		1.91	
	381	383*	1.97	1.96*
	387		1.99	
Pearled barley	332		2.39	
	325	319	2.21	2.30
	300		2.31	
Water-treated	394		1.79	
pearled barley	401	388*	1.75	1.78*†
	368		1.80	

* Significantly better ($P < .01$) than untreated pearled barley.

† *Idem* than corn.

Results. Table I shows outline of experiments and the results obtained on chick growth and feed efficiency. The values are given for replicate groups and also for treatment averages. The improvement in nutritional value of pearled barley by water treatment was very striking in both experiments. The results show that treated pearled barley was equal to corn for supporting chick growth. Feed efficiency for the diet containing treated pearled barley was significantly better than for corn ($P < .01$). Both diets gave significantly better growth and feed efficiency than the diet containing untreated pearled barley. The data were analyzed by Duncan's Multiple F test(4) and by the modification of Kramer(5).

Discussion. The marked improvement in nutritional value of pearled barley by the water treatment described was probably caused by increased availability of energy. Previous results(1) have shown that addition of tallow to similar diets containing untreated pearled barley gave chick growth and feed efficiency almost comparable with a diet containing corn. The possibility that an inhibiting substance was destroyed by the treatment cannot be eliminated, since appropriate experiments have not been conducted.

Summary. The results obtained in 2 independent experiments demonstrated that a simple water treatment of pearled barley markedly improved the nutritional value of this cereal grain. Treated barley was equal to corn for chick growth. The diet containing treated pearled barley gave significantly better ($P<.01$) feed efficiency than the diet containing corn or untreated pearled barley.

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Immunogenicity of Poliomyelitis Vaccine Prepared with Ultraviolet Irradiation and Mild Heat.* (23184)

Howard J. Shaughnessy, Albert M. Wolf, Martha Janota. John Neal, Franz Oppenheimer, Albert Milzer, Henry Naftulin and Richard A. Morrissey
Michael Reese Research Foundation, Ill. Department of Public Health, University of Ill. College of Medicine and Dept. of Microbiology, Michael Reese Hospital, Chicago.

In previous reports(1,2) it was demonstrated that poliomyelitis vaccines produced by inactivation with ultraviolet irradiation alone or in combination with heat were safe and effective when used in human volunteers. In this paper, experiments showing effects of varying intensities of ultraviolet irradiation with and without subsequent exposure to mild heat on inactivation of poliomyelitis virus and on antigenicity of the resulting vaccine in animals, are presented.

Material and methods. The vaccines were prepared from poliomyelitis virus propagated in monkey kidney tissue culture. Some preliminary work was done with vaccines made from virus grown in our laboratories; however, the bulk of the virus preparations used to prepare vaccines for this study was supplied by Parke, Davis and Co. and contained Mahoney (Type I), MEF-1 (Type II), and Saukett (Type III) strains. The material, except in one experiment, was irradiated as trivalent pools after preliminary studies had demonstrated that all 3 types of virus were essentially equally susceptible to ultraviolet irradiation, as shown in Table I. Just prior to initial irradiation the virus suspensions were filtered through a series of sintered glass filters (coarse, medium-fine and ultra-fine). In cases where 2 irradiations were employed in series (see below), an intermediate fine sintered glass filter was used. The irradiation was carried out in centrifugal filmers described in detail elsewhere†(3). Two filmers were connected in series in such a manner that samples could be taken after the virus suspensions had passed through one or both of them. The quantity of ultraviolet energy absorbed/ml of virus suspension was varied by passing the respective materials through the machine(s) at different rates. While exposure time is relatively constant, the net biological effect produced by irradiation is dependent upon a number of factors, discussed elsewhere†(1,3). At a flow rate of 200 ml/minute in the particular machine used, the film thickness is about 18 μ. An increase in flow rate results in an increase in film thickness, thereby reducing the quantity of energy absorbed per unit volume irradi-

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† To be published.

TABLE I. Titers of Poliomyelitis Virus Strains before and after Ultraviolet Irradiation.*

Virus strain irradiated	Titer† before irradiation	Titer after irradiation‡ at 25 watts	20 watts	15 watts	10 watts
Type I (Brunhilde)	6.8	1.2	1.1	3.1	4.4
I (Parker)	6.7	.6	.9	2.6	4.1
II (MEF)	6.8	1.0	1.5	3.5	4.4
III (Saukett)	7.0	1.1	1.5	3.0	3.7

* Table based on several experiments with each strain of virus, using 0.5 ml inoculation per tube of tissue culture, 10 tubes per level, log dilution series.

† $TCID_{50}$ (0.5 ml inocula) in monkey kidney tissue tube cultures.

‡ Irradiation in Centrifilmer at rate of 600 ml/min.

TABLE II. Irradiation Titration-Poliomyelitis Virus in 199 Fluid.

	Irradiation technic			Post irradiation titration				
						Tubes*		Bottles†
Initial titer	ml/min.	Watts	No. machines	Undil.	10^{-1}	10^{-2}	10^{-3}	
7.1	200	20	2	0/30				0/5
6.0	"	"	2	0/20				0/3
7.9	400	"	2					0/5
7.2	600	"	2	0/10				3/3
6.0	"	15	2	0/10				3/3
6.1	800	20	2	0/10				2/5
6.2	1,000	"	2	2/10	0/10			1/1
7.9	400	"	1	3/ 5	2/10	0/10		
6.1	800	"	1	10/10	7/10	1/10	0/10	
7.2	600	"	1	10/10	10/10	4/10		
6.2	1,000	"	1	5/ 5	5/ 5	3/ 5	0/ 5	
6.0	600	15	1	10/10	5/ 5	2/ 5	1/ 5	

* 0.5 ml inocula/tube. † 50 ml inocula/32 oz bottle.

ated. Flow rates of from 200 to 1,000 ml/minute were employed and the virus suspension was passed through either 1 or 2 machines at these rates. The titers of the resulting irradiated suspensions are shown in Table II. In most studies here reported, 50 ml/inocula/bottle were used in the safety tests. Subcultures were made from each bottle to at least 10 tubes of tissue culture after the bottles had been incubated 7 and 14 days, respectively. Since no chemicals were added, it was not necessary to dialyze the irradiated samples prior to performing safety tests. Potency tests of vaccines were made in mice and monkeys by the methods described in "Minimum Requirements: Poliomyelitis Vaccine"—1st Revision, April 12, 1955, by the Nat. Inst. H. Antibody titrations of the animal sera were made in monkey kidney tissue cultures, using the roller tube technic(4) or the metabolic inhibition procedure(5). Our own standard reference serum was used in most of these titrations. Titers are expressed in terms of the 50% end point of serum dilution in all cases.

Results. Table III gives the results of potency tests in mice or monkeys and safety tests on lots of vaccines prepared by irradiation alone. The mouse potency results shown in this Table and those in Table IV are the titers in mice which had received the undiluted vaccine. The monkey potency results are the arithmetic means of the titers of individual monkey serums.

It would appear from the results shown in Table III that the virus suspensions exposed at the rate of 200 ml/minute may have been over-irradiated to a point where antigenicity was impaired. However, increasing the flow rate in each of 2 machines to 400 ml/minute did not appreciably improve potency. With flow rates of 600 ml/minute or higher residual live virus was found when 150-250 ml amounts of vaccine were tested in tissue culture regardless of the wattage employed.

It was then decided to try a combination of irradiation and mild heat to produce inactivation. Table IV gives the results of safety and potency tests of experiments in which combinations of ultraviolet energy, as established

TABLE III. Immunogenicity of Vaccine Inactivated by Ultraviolet Irradiation Alone.

Exp. No.	Trivalent titer, pre-irrad.	Flow rate, ml/min.	Exposure to ultraviolet, watts*	Post irrad. TC-titer		Mouse potency types			Monkey potency types		
				Tubes§	Bottles‖	I	II	III	I	II	III
1	7.1	200	20–20	0/30	0/5	8	8	256	2	19	3
Control†						4	8	8	0	0	0
5	6.0–6.7‡	”	”	0/20	0/3	16	16	4			
Control						8	8	8			
7	7.0	”	”	”	0/2	4	4	4			
Control						8	8	8			
11	7.9	400	”		0/5	4	64	4	18	45	5
Control						4	4	16			
12	7.9	600	”	0/10	3/3						

* 20–20 signifies that material was subjected to 2 sequential irradiations, with 20 watts of incident energy used in each irradiation.

† Undiluted formalinized control vaccine, supplied by Parke, Davis and Co.: Used in all experiments.

‡ Range of monovalent titers.

§ Tissue culture 0.5 ml/tube.

‖ ” ” 25-50 ml/bottle.

by flow rates through one or two filmers, are followed by holding the suspensions at 37-40°C for 3 to 20 days. The potency data do not allow an accurate comparison between the several experimental lots because the pre-irradiated suspensions were not identical in all essential respects, especially in titer. However, the results would suggest that the antigenic potential of the vaccines produced by inactivation with ultraviolet and heat compare favorably with the formalin control vaccine.

Virus suspensions which had been inactivated by exposure to irradiation and mild heat for short periods of time, such as 5 days at 37°C were tested for "reactivation" by exposure to mild heat for additional periods of 3 to 5 days. Reactivation did not occur under our conditions; indeed exposure to heat acts as an inactivating agent rather than as a reactivating process. It was also found that exposure to mild heat for as long as 5 days after active virus had disappeared, did not cause a significant reduction in antigenicity. This indicates that a margin of safety of several days' duration can be employed in the inactivation process without impairing antigenicity. There is evidence(6) that mild heat alone does not produce a killed poliomyelitis virus vaccine with satisfactory antigenicity. Apparently, marginal lethal levels of ultraviolet may leave some virus particles viable but injured and incapable of survival if held at temperatures of 37° to 45°C for a few days. On the other hand, virus particles remaining viable after irradiation are inactivated very slowly if placed at 5°C.

Three of the lots of vaccines were retested in animals after storage at 4°C. The results (Table V) indicate only slight antigenic deterioration in vaccines TV8 and TV9 after storage for 6 and 7 months, respectively. Type 1 and 3 components of vaccine TV7 seemed to have deteriorated somewhat more during a storage period of 10 months but there was still a fair amount of antigenicity present.

Discussion. The results presented give capacity of ultraviolet irradiation to destroy cytopathogenic capacity of polio virus under certain experimental conditions. It was found that, if sufficient ultraviolet energy was used to destroy completely the cytopathogenicity of the virus for monkey kidney cells, the antigenic potential was reduced. However, as was shown previously(1), such vaccines still retain the capacity to induce an immune response in humans.

Since the safety tests used to establish the non-cytopathogenicity of inactivated polio virus have been made more critical(7), it appeared reasonable to attempt to use combinations of inactivation procedures. Inactivation of viruses by means of ultraviolet irradiation alone is a physical method and, logically, the

TABLE IV. Immunogenicity of Vaccine Inactivated by Ultraviolet Irradiation and Mild Heat.

Exp. No.	Trivalent titer, pre-irrad.	Flow rate, ml/min.	Exposure to ultraviolet, watts*	Days of mild heat 37°C	Days of mild heat 40°C	Safety test after mild heat†	Mouse potency types I	Mouse potency types II	Mouse potency types III	Monkey potency types I	Monkey potency types II	Monkey potency types III
11	7.9	400	20	5		0/1	16	64	8	44	93	69
		”	20–20	5		0/3	4	64	4	4	102	8
		”	”	10			4	32	4	4	74	4
Control‡							4	4	16			
12	7.9	600	20	10		0/2	16	64	32	18	525	212
		”	20–20	5		0/5	32	32	64	19	131	11
Control§							4	4	32	65	290	130
” ‖							64	32	256			
14	6.1	800	20	20		0/5	8	32	32			
		”	20–20	5		”	8	32	32	8	35	1
		”	”		3	0/2	8	16	16			
Control¶							4	8	16	12	64	4
16¶	6.0	600	20		10	0/5	64	128	128			
		”	10–10		10	”	64	128	64			
Control¶							8	64	128			
17¶	6.2	600	20		10	0/5	64	128	64			
		”	20–20		3	”						
		”	”		5	”						
		”	”		8	”	16	256	64			
Control¶							16	64	128			
18¶	7.1	600	20–10		7	0/45	16	16	32			
Control¶							16	64	32			

* Each individual number signifies incident energy of irradiation; single number indicates one irradiation; double number, 2 irradiations; see footnote * Table III.

† In tissue culture bottles, 50 ml per bottle: Bottles negative cytopathogenically/bottles inoculated.

‡ Undiluted formalinized vaccine, supplied by Parke, Davis and Co., unless otherwise indicated, used in all experiments.

§ Control vaccine (‡) diluted 1:4.

‖ Live virus suspension diluted 1:5.

¶ Both test vaccine and control vaccine (‡) diluted 1:4.

combination of ultraviolet irradiation and another physical method, incubation, was studied in these experiments. The results indicate that exposure of virus suspensions to ultraviolet energy sufficient to reduce the initial titer 4 to 6 logs, followed by incubation, appears to produce satisfactory vaccines.

The reasons for the effectiveness of the ultraviolet-mild heat combination in producing a non-cytopathogenic suspension are not entirely clear. It may be that secondary inactivation is responsible and that this proceeds more rapidly at temperatures of 37° to 45°C. An alternative hypothesis is that virus particles which are altered(8) or injured by the ultraviolet, but not completely killed, more rapidly become non-cytopathogenic at the incubator temperatures. Of course, other explanations are possible, and

TABLE V. Stability of Vaccines Stored at 4°C. Antibody titers (mouse).

Vaccine No.	Months of storage	Dilution of vaccine	Virus type I	Virus type II	Virus type III
TV7	0	Undil.	32	32	64
		1: 5	16	4	16
		1:25	0	4	8
	10	Undil.	8	32	16
		1: 5	4	4	16
		1:25	0	0	0
8	0	Undil.	8	32	32
		1: 5	8	16	8
		1:25	8	16	4
	7	Undil.	16	32	32
		1: 5	4	16	4
		1:25	0	0	0
9	0	Undil.	8	32	16
		1: 5	4	4	4
		1:25	4	4	4
	6	Undil.	16	32	32
		1: 5	0	8	16
		1:25	0	0	0

subsequent work in progress is designed to explore them.

Summary. 1. Poliomyelitis virus of high titer in the form of a freshly filtered tissue culture preparation, can be inactivated by ultraviolet irradiation in centrifugal filmers. 2. Inactivation of the virus preparations by a combination of ultraviolet irradiation and exposure to mild heat for several days yields vaccine of good potency. In this procedure there is a margin of safety between complete inactivation and appreciable loss of antigenicity. 3. Experience thus far indicates a degree of consistency and reproducibility for the method described. 4. Vaccines made by a combination of ultraviolet irradiation and mild heat appear to retain their antigenicity for at least 7 months.

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Bruised Tissue. I. Biochemical Changes Resulting from Blunt Injury.*

(23185)

MOSTAFA K. HAMDY, FRED E. DEATHERAGE, AND GEORGE Y. SHINOWARA

Departments of Animal Science, Agric. Biochemistry, and Pathology, Ohio State University, Columbus, and Ohio Agric. Exp. Station, Wooster.

A bruise is a tissue injury without laceration usually produced by a blunt object resulting in rupture of the vascular supply and accumulation of blood and fluid. The accumulated blood undergoes, during the healing process, various physical and biochemical changes, represented by the color alteration characteristic of a bruise. Williamson(1) stated that the biochemical studies on wounds and wound healing have been devoted to the metabolism by the injured organism and the determination of the character and metabolism of certain proteins and polysaccharides of the wound area. In this report studies were undertaken to investigate the nature of some of the physical and biochemical changes occurring during the healing process of a bruised tissue.

Materials and methods. Bruising and sampling. Fifty-five cattle were used. The areas to be bruised and symmetrically located control areas were clipped and shaved. Injury was inflicted by 2 blows of a 7 pound sledge hammer over 3 feet in one-half second. (The degree of injury inflicted experimentally was less than that often sustained by cattle due to falls or contacts with other animals and sides of vehicles while in transit.) Observations were made of the bruised area for 9 days after injury. At various intervals animals were slaughtered and the bruised and control areas were excised, sliced, minced, and subjected to various chemical and physical studies. *Easily split iron* (E.S.I.). The tissue sample was homogenized with 0.4% HCl and incubated 24 hours at 37°C(2) and the E.S.I. was determined as the ferrous compound of o-phenanthroline(3). *Pigment extraction.* Saline, buffered to pH 7.4 and con-

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TABLE I. Iron* and Non-Protein Nitrogen Contents of Bruised Cattle Tissue as Related to Gross Appearance and Time.

Age of bruise	Appearance				No. of samples	mg/100 g tissue‡	
	External		Fluid				
	Swelling	Color	Vol	Color		Iron	N.P.N.
Control†	—	Normal	—	Normal	30	.25 ± .07	650 ± 18
15 min.	++	Dark red	++	Red	4	.30 ± .03	560 ± 20
2 days	+++	*Idem*	+++	Dark red	4	.32 ± .04	460 ± 15
3	++	Light green-purple	++	Brown-yellow	5	.46 ± .05	410 ± 13
4	+	Yellow-green-purple	+	Yellow-orange	3	.50 ± .04	320 ± 12
5	—	Orange	±	*Idem*	5	.57 ± .05	140 ± 8
6	—	Slight orange	±	Slight yellow	3	.32 ± .04	200 ± 12
7	—	Normal	—	—	3	.25 ± .06	240 ± 10
8	—	”	—	—	2	.27 ± .05	500 ± 18
9	—	”	—	—	2	.32 ± .03	720 ± 16

* “Easily split iron.”

† Control (normal) tissues from symmetrically located areas as the bruises.

‡ Mean ± S.D.

taining 1% normal human serum albumin, was used for extraction of some of the pigments present in the bruised and control tissues, followed by chloroform extraction. *Hemoglobin and bilirubin.* The American Optical Rapid Scanning Spectrophotometer was used for qualitative studies of these pigments in saline and chloroform extracts. The concentrations of hemoglobin and bilirubin in the saline extract were determined(4) using a Beckman D.U. spectrophotometer. Bilirubin concentration in the chloroform extract was determined spectrophotometrically at 450 mμ. *Non-protein nitrogen (N.P.N.).* A modification of the Miller and Miller(5) procedure was used for determination of the N.P.N. in the saline extract.

Results. In Table I are recorded observations of bruises inflicted on cattle using the technic described. It is evident that the mean concentration of iron (E.S.I.) in bruised tissue rose immediately up to and over the control level (0.25 mg %) in 4-5 days after injury and then decreased to reach the control level within 7-9 days, at which time the bruise had apparently healed. The non-protein nitrogen, on the other hand, decreased from the control level of 650 mg% to 140 mg% in 5 days, but rose abruptly to the control value in 9 days.

Of the total pigment in exsanguinated normal muscle tissue, myoglobin is quantitatively the most important(6), whereas hemoglobin represents only approximately 10% (7,8). Therefore, the data given in this paper for normal (control) tissue are for both myoglobin and hemoglobin. Fig. 1 represents typical spectrophotometric absorption curves of saline extracts of normal and of 5-day-old bruised tissue samples. Both extracts differ in several ways. The greater absorption at 560 and 575 mμ (corrected for absorption curve of the didymium glass standard) of bruised tissue extract than that of normal tissue, is attributed to increase in hemoglobin concentration. The plateau at 600-640 mμ in bruised tissue extract suggests the presence of alkaline hematin which is absent in the control extract. The absorption spectrum of a pure bovine hemoglobin is shown in Fig. 2 for comparative purposes.

After the presence of hemoglobin in the bruised tissue was established it was decided to follow quantitatively its changes during the healing process. The results (Table II) indicate that extra-stromal hemoglobin concentration increased immediately following bruise infliction, followed by a decrease to reach the level of normal tissue upon complete healing. It appears therefore that any increase in red pigment in bruised tissue compared to that of the control, is due primarily to hemoglobin and traces of hematin. Apparently there is very little change in myoglobin concentration due to bruising.

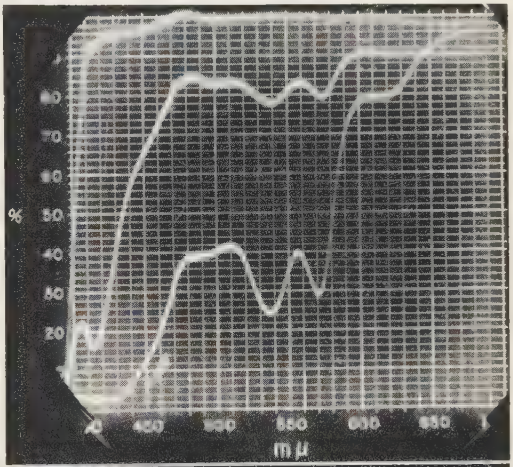
The concentration of the yellow pigments,

TABLE II. Relation between Extra-Stromal Hemoglobin and Bilirubin Concentrations in Bruised Cattle Tissue.

	Concentration in mg/100 g tissue					
	Myoglobin and hemoglobin		Yellow pigments as bilirubin			
			Total		Bilirubin	
Age of bruise	Control	Bruised	Control	Bruised	Control	Bruised
15 min.	52.5	180.2	.76	.43	.00	.00
2 days	42.4	228.9	.82	2.28	"	1.40
3	40.6	425.2	.45	4.17	"	3.52
4	60.2	500.2	.86	4.30	"	3.89
5	57.5	372.0	.67	2.36	"	1.90
7	71.8	390.6	.32	1.80	"	1.39
8	78.2	80.5	.50	2.20	"	1.30
9	60.2	68.2	.40	.50	"	.20

representing the total of that found in both the saline and chloroform extracts, was then determined spectrophotometrically with C. P. bilirubin (Eastman) as a standard. The results obtained on control and bruised tissues are recorded in Table II. It can be seen that total yellow pigments in normal tissue ranged from 0.32 to 0.86 mg/100 g, while that of bruised tissues increased to 4.3 within 4 days of injury, followed by a rapid decrease, to reach the normal tissue level. Total bilirubin was then determined chemically by the van den Bergh reaction on the saline and chloroform extracts of control and bruised tissue: The results are shown in Table II. It is evident that control tissue extract did not contain bilirubin, *per se,* while its concentration in bruised tissue extracts rose to 3.8 mg/100 g tissue within 4 days after injury and then decreased to 0.20 mg/100 g by the ninth day. Therefore, the increase in total yellow pigments due to injury is attributed to bilirubin. This was confirmed by isolating the pigment as the barium salt: The free pigment had a maximum absorption at 450 mμ, identical to that of C. P. bilirubin. The yellow pigments in the normal (control) tissue must be substances other than bilirubin such as lipochromes, riboflavin and hemoglobin, all of which absorb light at 450 mμ.

Discussion. The increase of extra-stromal hemoglobin during early stages of healing of bruised tissue apparently resulted from destruction of red blood cells which have escaped from the circulation upon rupture of the capillaries. This increase of extra-stromal hemoglobin occurred along with an increase in iron (E.S.I.) content of bruised tis-



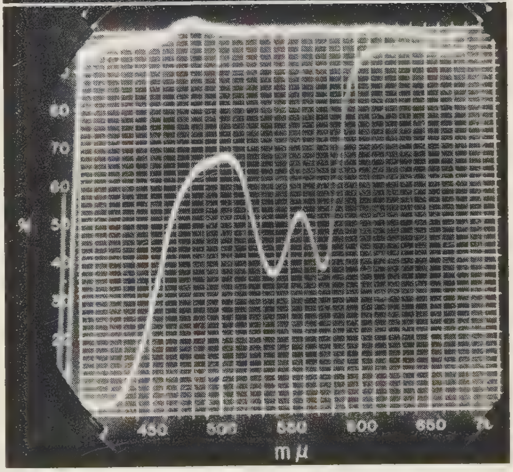


FIG. 1 (top). Spectrophotometric absorption curves of saline extracts of a 5-day-old bruise in cattle and a control (non-bruised) tissue from a symmetrically located area, first (top) curve, saline reference; second curve, control extract; third (bottom) curve, bruise extract.

FIG. 2 (bottom). Spectrophotometric absorption curves of bovine hemoglobin. First curve, saline reference; second curve, hemoglobin solution, approximately 70 mg/100 ml saline.

sue, indicating that this hemoglobin was catabolised in the tissue by release of its iron. No explanation is given for the drop of the N.P.N. in early stages of healing, but the higher concentration of this N.P.N. after 4-5 days may be due to excessive tissue catabolism and degradation of the globin moiety of the hemoglobin molecule. Selye and Dosne (9) found that blood which passed through a damaged area contained a high concentration of free hemoglobin and N.P.N. The absence of bilirubin immediately after bruising the tissue and its rapid increase in 3 or 4 days followed by rapid decrease in concentration seem to indicate that this pigment is a degradation product from the catabolism of free hemoglobin. It should be stated that increase of bilirubin concentration coincided with increase of the orange color usually observed during the healing process of a bruise. The detection of hematin (Fig. 1) in the bruised tissue during healing seems to indicate that globin is first split off from the hemoglobin molecule giving rise to hematin. The latter loses its iron and appears to undergo oxidation resulting in the opening of the tetrapyrrolic ring of the molecule in the α-position with loss of α-methyne bridge ($-CH=$) and formation of 2 hydroxyl groups at the end of the linear tetrapyrrolic molecule. It is possible that biliverdin is the first compound formed which may be reduced by the reducing system of the tissues to form bilirubin as indicated by Lemberg and Wyndham(10). These physical and biochemical changes during healing of bruised tissues were also investigated in 100 rabbits and the results followed the same pattern. The sequence of events resulting in the degradation of hemoglobin during healing of a bruised tissue is consistent with the data of *in vitro* experiments reported by Lemberg and Legge(11).

Summary. In experimentally inflicted bruises of cattle, the greatest swelling and fluid volume occurred within 2 days. The maximum biochemical changes in bruised tissue were found on or about the fourth or fifth day: These included a drop in N.P.N. to one-fourth of the control level and a 2-fold increase in the "easily split iron" concentration. Moreover, the 10-fold elevation in red pigment was due to extra-stromal hemoglobin. Bilirubin formation in bruised tissue has been demonstrated conclusively and the probable metabolic derivation of this pigment is discussed. Although healing was evident grossly in 7 days, the biochemical values did not return completely to the control levels until the ninth day.

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Gastrointestinal Absorption with Oleic Acid-I^{131} and L-Methionine-S^{35} in Patients with Ulcerative Colitis.* (23186)

David J. Sandweiss and Stanley H. Levy
Division of Medicine, Sinai Hospital, Detroit, Mich.

Several studies have been reported pointing to possible impairment of small bowel absorption in patients with ulcerative colitis (1-5). These studies employed the closed intestinal loop technic or tolerance tests, or dealt with percentage loss of nitrogen in the ileal discharge. As to the increased outputs of nitrogen and of amino acids in the feces of patients with ulcerative colitis, (with intact colons), several observers(6-8) suggested that the increase appears attributable to excess exudate and blood from the colon rather than to unabsorbed proteins. Ruffin *et al.*(9) studied 8 patients with ulcerative colitis using an I^{131} labeled fat (glycerol trioleate). They reported that the average values of the blood were well within normal limits in each of the 8 patients, as was also the radioactive fat material in the stool.

In the present study we employed oleic acid-I^{131} and l-methionine-S^{35} as test material for absorption. By use of basic food substances we minimized, or eliminated completely, the digestive influence required with whole food substances. As noted previously (10) our data suggest that patients with ulcerative colitis might have impairment of absorption of oleic acid and methionine, more especially methionine.

Method. All subjects were hospital inpatients, except 2 patients with ulcerative colitis during stage of remission. Control subjects (16 subjects with 17 studies) were free from any gastrointestinal disease. The ulcerative colitis patients (21 patients with 54 studies) were studied during different phases of the disease. In certain instances the same patient was studied during acute and mild phases of their illness and during remission. Six patients were studied with each tracer substance following ileóstomy and colectomy. During active stages of the disease the colitis patients were on standard supportive therapy (low residue, high caloric, high protein diet; antispasmotics or anticholinergic drugs; sedatives; azulfidine; vitamins). Five patients were also on steroid therapy. The ileostomy-colectomy patients as well as those patients studied during the phase of clinical remission received no medication. This report deals with 71 studies in 37 subjects.

Oleic acid-I^{131} was administered in doses between 5 and 18 μc (usually 5-10) and l-methionine-S^{35} in doses between 18 and 40 μc (usually 18 to 22) dissolved in alcohol and water, respectively. These compounds were given at an interval of not less than 3 days, with methionine-S^{35} being given first. The test substance was administered orally at approximately 8 a.m. on an empty stomach. The patients were fed a standard breakfast from 2 to 6 hours (usually 4 hours) after administration of test material. Each fecal sample was collected in individual container and in earlier studies quantity of radioactive material was determined in each sample. In later studies, feces for each 24 hour period through 72 hours was pooled and then analyzed for activity. These specimens were diluted with sufficient water, homogenized with a Waring blender so that a roughly constant specific gravity was obtained (sp. gr. 1.005-1.010). After noting the obtained volumes 3 ml aliquot was taken when counting I^{131} activity and 2 ml aliquot when counting S^{35} activity. A standard, equal to the amount given, was set up for each patient and appropriate calculations were made to determine percent of dose in each specimen. Individual percentages were totaled to give the 72-hour figures. I^{131} activity in liquids was determined in a well-type scintillation counter for 3 minutes while S^{35} activity in liquids was determined with a thin-window TGC-2 Geiger tube for 10 minutes, utilizing constant

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* This study was done in cooperation with the Isotope Laboratory of the Department of Radiology in charge of Dr. Howard H. Feigelson

TABLE I. 72-Hour Fecal Excretion of I^{131} and S^{35} following Administration of Oleic Acid-I^{131} and Methionine-S^{35}. Control subjects and patients with ulcerative colitis. (% of orally administered dose.) 71 studies.

	I^{131}						S^{35}					
	No. of patients	No. of studies	Avg	Range	t*	p*	No. of patients	No. of studies	Avg	Range	t*	p*
Control subjects	9	9	5.4	4.2- 6.9			8	8	5.6	4.0- 7.0		
Ulcerative colitis												
Acute phase	6	8	12.5	7.6-20.3	3.797	<.01	7	9	39.7	23.5-64.9	6.953	<.001
Mod. activity	4	4	8.5	6.3-10.2	2.43	<.05	7	7	21.8	12.4-27.6	7.869	"
Remission	6	6	7.3	4.7- 9.9	1.89	>.05	7	7	15.0	10.7-20.0	6.88	"
Ileostomy-colectomy	6	7	13.2	5.4-24.5	2.878	<.05	6	6	26.1	17.7-31.4	9.951	"

* "t" is ratio of mean to stand. error; "p" is probability of occurrence of an event. (See also Batson, H. C., *An Introduction to Statistics in the Medical Sciences*. Burgess Publishing Co., 1956.)

geometry.

Results. 1. The data on fecal excretion are summarized in Table I. In the control subjects the fecal fraction of the administered dose was approximately the same for both I^{131} and S^{35}. 2. Fecal excretion of I^{131} was increased during the acute phase of ulcerative colitis as well as during the period of moderate relapse. The differences between these 2 groups of patients and control subjects are statistically significant. However, the difference in results between patients in remission and the control subjects is not statistically significant. 3. Fecal excretion of S^{35} was increased during the acute phase of ulcerative colitis. As the patients improved clinically, the excretion of S^{35} returned toward control values only slowly so that even during the remission stage, average fecal excretion of S^{35} was still more than twice the average control level. The differences in the results between each of the 3 groups of patients and that obtained in the control group are statistically highly significant. 4. In 6 patients following ileostomy and colectomy, radioactivity of I^{131} in ileal fecal excretion fluctuated considerably. The difference in results is statistically significant. However, in studies with S^{35} ileal fecal excretion was consistently increased and the difference is statistically highly significant. Five of the 6 patients were in excellent health, with the ileostomy and colectomy having been performed from 19 to 58 months prior to the isotope studies; the 6th patient was still convalescing from the operation which was performed 3 months previous to the study.

Table II summarizes results obtained in 5 colitis patients, each of whom was studied during different stages of the disease. The 72-hour fecal S^{35} excretions decreased as the patients improved clinically. However, fecal radioactivity values failed to reach, at any time, the values obtained in control subjects. Such a consistent pattern was not observed with I^{131}.

Summary. Following oral administration of oleic acid-I^{131} patients with ulcerative colitis showed increased fecal excretion of I^{131} during the acute phase of the disease, but it returned toward control values as the pa-

TABLE II. I^{131} and S^{35} Outputs in 72-Hour Stools in 5 Patients with Ulcerative Colitis Studied during Different Stages of the Disease with Oleic Acid-I^{131} and Methionine-S^{35}.

Patient	Date of study	Stage of disease	% test dose	
			I^{131}	S^{35}
S.C.	* 1/13/56	Acute	7.6	39.9
	* 3/ 6	Convalesc.	9.6	17.6
	4/15	Remission		13.7
	*11/29	Acute	13.0	64.9
	* 2/26/57	Ileost.-colec.	12.1	17.7
C.P.	* 9/16/56	Mod. activity	6.3	23.6
	*12/ 1	Remission	9.9	12.5
H.J.	* 6/21	Acute	7.8	29.6
	*12/ 1	Remission	5.1	20.0
P.K.	3/12	Acute	20.3	
	7/ 1	"		57.1
	* 1/29/57	Remission	8.8	13.6
J.L.	11/ 2/56	Acute		51.6
	1/31/57	Remission		17.6
	3/ 1	"	10.9	

* In these patients the methionine-S^{35} study was begun on the specified date and the 72-hr study with oleic acid-I^{131} was begun 4 days later.

tients improved clinically. Following administration of methionine-S^{35}, fecal excretion of S^{35} increased during the acute phase of the disease to a greater degree than the excretion of I^{131}. Also, as the patients improved clinically excretion in the feces returned toward control values only slowly, with the result that during the remission stage of the disease excretion of S^{35} was still elevated and it was elevated in all ulcerative colitis patients studied to date including those with ileostomy and colectomy.

It is a pleasure to express our gratitude to Mrs. Madeline E. Ranney for her technical assistance.

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Multiplication of Eastern Equine Encephalitis Virus in Mosquitoes Following Intrathoracic Inoculation.[*] (23187)

ROY W. CHAMBERLAIN AND W. D. SUDIA (Introduced by Morris Schaeffer)
Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare, Montgomery, Ala.

Infection of mosquitoes with arthropod-borne encephalitis viruses by intrathoracic inoculation has been demonstrated(1,2).[†] Because minimal concentrations of viruses are sufficient to infect mosquitoes by this route, this method appears to be excellent for studying rates of virus development within mosquitoes, uncomplicated by chemical or physical barriers to infection encountered in the digestive tract.

In the present study the intrathoracic inoculation technic was used to determine rate of multiplication of eastern equine encephalitis (EEE) virus in *Aedes triseriatus* mosquitoes.

Methods. The mosquitoes used were *Aedes triseriatus* 3 to 6 days old. They were anesthetized by 15 to 20 seconds' exposure to carbon dioxide gas immediately prior to inoculation. Method of inoculation was similar

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[*] This work was supported by contract with the Chemical Corps, Ft. Detrick, Frederick, Md.

[†] L. Whitman, personal communication.

TABLE I. Multiplication of EEE Virus in *A. triseriatus* Mosquitoes following Intrathoracic Inoculation.

Hrs incubation	Mouse i.c. LD_{50} recovered/mosquito following inoc. with: 300 mouse LD_{50}	.3 mouse LD_{50}
0	50 - 300	<30
8	300 - 1,250	<30
18	1,600 -16,000	<30
26	16 - 160*	30 - 800
42	63 - 1,000*	16 - 200*
66	200 - 3,000*	60 - 600*
90	160 - 1,000*	50 -1,600*
168	200 - 1,000*	300 -2,000*

* × 1000.

to that of Whitman.† A piece of 4 mm glass tubing was drawn out in a flame, then the drawn section was heated in a tiny flame and drawn out again. The tip was snapped off at approximately 75 μ diameter. A piece of rubber tubing was attached to the undrawn end and infected fluid was drawn into the capillary by sucking on this rubber tubing. The fluid was ejected after insertion of the capillary tip into the side of a mosquito thorax by clamping the end of the rubber tube and squeezing the mid-part of the tube in the hand. The EEE virus strain used had been isolated from wild-caught mosquitoes(3) and had undergone 2 mouse brain, 1 chick embryo, and 7 serial mosquito intrathoracic passages. Individual mosquitoes in 2 groups were inoculated intrathoracicly with approximately 0.0003 ml each of either a 10^{-1} or 10^{-4} dilution of EEE-infected mosquito suspension. The 10^{-1} inoculum contained approximately 300 mouse intracerebral LD_{50}, and the 10^{-4} inoculum, about 0.3 mouse intracerebral LD_{50}. Five mosquitoes of each group were then incubated at 80°F and 75% relative humidity for the following periods: 0 hr, 8 hrs, 18 hrs, 26 hrs, 42 hrs, 66 hrs, 90 hrs, and 168 hrs. After incubation they were killed by freezing, ground individually in 1 ml of 25% normal horse serum in buffered water, pH 7.7, and titrated by intracerebral inoculation of .03 ml amounts into 3-week-old white Swiss mice. The amount of virus in each mosquito was calculated to be 33 times the titer of the 1 ml mosquito suspensions.

Results. The results are given in Table I. Apparently nearly a 10-fold increase of virus occurred each 8 hours. About 8 to 9 eight-hour periods were required for the dilute-inoculum series to reach peak virus levels of approximately 1,000,000 - 2,000,000 mouse LD_{50} units per mosquito, while the concentrated-inoculum series required only about 5 of these periods. The nearly straight-line relationship between rates of virus multiplication in the dilute- and concentrated-inocula groups suggests that no appreciable initial lag period followed inoculation and that multiplication occurred cyclically. Recovery of amounts of virus varying from 50 to 300 mouse LD_{50} from mosquitoes immediately following inoculation of an estimated 300 LD_{50} shows close approximation of dosages given.

Summary. Known amounts of eastern equine encephalomyelitis virus were inoculated into the thorax of *Aedes triseriatus* mosquitoes. Individual mosquitoes were ground and tested quantitatively for virus content after various periods of incubation. Approximately a 10-fold increase in virus occurred every 8 hours until maximum virus levels were attained.

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Comparative Effects of Alpha-Tocopherol, DPPD and Other Antioxidants on Muscular Dystrophy in Guinea Pig.* (23188)

R. SHULL, R. B. ALFIN-SLATER, H. J. DEUEL, JR.,† AND B. H. ERSHOFF
Department of Biochemistry and Nutrition, University of Southern California, Los Angeles.

Considerable data are available indicating that various antioxidants unrelated chemically to the tocopherols can prevent various manifestations of vit. E-deficiency in experimental animals. Methylene blue, thionine and thiodiphenylamine, for example, were all active in promoting growth and preventing exudative diathesis in chicks fed a diet deficient in vit. E(1). Methylene blue and DPPD (N, N′-diphenyl-p-phenylenediamine) prevented encephalomalacia in chicks(1,2) and resorption gestation in rats(3-5) fed diets deficient in vit. E. Methylene blue was also active in preventing dystrophy in calves fed a diet containing cod-liver oil(6) while DPPD showed similar activity in preventing symptoms of stiff lamb disease and histological degeneration of the skeletal and cardiac muscles of lambs fed a diet deficient in vit. E(7). There is evidence to indicate, however, that the above antioxidants will not completely replace vit. E in the diet but that the apparent tocopherol-like potency of these materials is due to their sparing effect on small residual quantities of vit. E in the diet and/or the body tissues. This is indicated by findings that the above antioxidants were devoid of vit. E activity as judged by the prevention of fetal resorptions when the diet was rigorously freed of tocopherol and the depletion period was extended(8,9). In the present communication further data are presented on the comparative effects of alpha-tocopherol and various antioxidants on the incidence and time of onset of muscular dystrophy in guinea pigs fed a highly purified diet deficient in vit. E.

Procedure. The basal vit. E-free ration employed consisted of casein,‡ 30%; cornstarch, 20%; dextrose, 11%; sucrose, 10%; cellulose,§ 10%; salt mixture,‖ 6%; lard,¶ 5%; agar, 5%; potassium acetate, 2.5%; and magnesium oxide, 0.5%. To each kg of the above diet were added the following vitamins: thiamine hydrochloride, 16 mg; riboflavin, 16 mg; pyridoxine hydrochloride, 16 mg; calcium pantothenate, 40 mg; nicotinic acid, 200 mg; biotin, 1 mg; folic acid, 10 mg; vit. B_{12}, 100 μg; 2-methyl-naphthoquinone, 5 mg; para-aminobenzoic acid, 100 mg; inositol, 2 g; choline chloride, 2 g; vit. A, 5000 U.S.P. units and vit. D_2, 500 U.S.P. units. The vitamins were added in place of an equal amount of cornstarch. Each guinea pig also received daily (6 times/week) an oral supplement of 10 mg ascorbic acid in 0.1 ml water. In preparation of the diets the various ingredients (minus the agar and the lard) were thoroughly mixed. The agar was dissolved in hot water (approximately 400 cc water/kg of dried diet), and the agar-water mixture was then mixed with the rest of the diet after which the lard was added. The mixture was then strained through an electric meat grinder and dried in an oven at a temperature of 110° to 120°F. In addition to the basal vit. E-free ration, tests were also conducted with animals fed the basal ration plus the following antioxidants: (1) 0.025% DPPD (N, N′-diphenyl-p-phenylenediamine), (2) 0.025% Santoquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline), and (3) 0.025% DBH (2,5-di-tert-butylhydroquinone). The above supplements were incorporated in the diet in place of an equal amount of cornstarch. Sixty

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† Deceased April 17, 1956.

‡ Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

§ Solka-floc BW 200, Brown and Co., Berlin, N. H.

‖ Salt Mix Wesson Modified (Osborne-Mendel), General Biochemicals, Chagrin Falls, O.

¶ Stripped Lard, Distillation Products Industries, Rochester, N. Y. Tocopherol content of this material according to manufacturers is less than 5 μg/g.

female guinea pigs were selected, average body weight 224 g (range 204 to 257 g). Animals were divided into 5 comparable groups of 12 guinea pigs each. One group was fed the basal ration; a second group received basal ration plus an oral supplement of 5 mg α-tocopherol acetate/animal (in 0.1 ml propylene glycol) 6 times weekly; the remaining 3 groups were fed the diets containing DPPD, Santoquin and DBH. Animals were placed in metal cages with raised screen bottoms (2 animals/cage) and were fed the above diets and water *ad libitum* for 1 year or until death, whichever occurred sooner. Diets were prepared biweekly and were stored under refrigeration when not in use. Animals were fed on alternate days. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet.

Results. For the first 12 weeks of feeding no significant differences in weight or gross appearance were observed between the various groups. All animals survived and were indistinguishable grossly from guinea pigs of a similar age which had been fed a natural food stock ration. The average body weight after 12 weeks of feeding on the various diets was as follows: basal ration, 506 g; basal ration plus alpha-tocopherol acetate, 537 g; basal ration plus DPPD, 534 g; basal ration plus Santoquin, 545 g; and basal ration plus DBH, 510 g. Animals of a comparable age on the natural food stock ration averaged 589 g. During the 13th week, 3 of the guinea pigs on the basal vit. E-free ration developed clinical symptoms of dystrophy (dragging of the hind limbs and delayed righting when the animals were placed on their back). At this time the 3 dystrophic animals and one non-dystrophic animal from this group and 4 guinea pigs from each of the other groups were autopsied and sections of the gastrocnemius muscle were prepared for histological study. The dystrophic animals exhibited the typical Zenker degeneration noted by Pappenheimer(10) and others as characteristic of the dystrophic voluntary muscles of vit. E-depleted animals. The musculature of the other animals appeared normal in all respects. In contrast to results obtained on the basal vit. E-free ration, animals fed diets containing DPPD, Santoquin or DBH did not develop clinical symptoms of dystrophy until after the 25th week of feeding, whereas no dystrophy occurred in any of the animals administered alpha-tocopherol acetate. Results are summarized in Table I.

TABLE I. Comparative Effects of Alpha-Tocopherol Acetate, DPPD and Other Antioxidants on Incidence and Time of Onset of Muscular Dystrophy in Guinea Pigs Fed a Vit. E-Deficient Ration (8 Animals/Group).

Supplements fed with basal vit. E-deficient ration	Incidence of dystrophy (%)	Avg time of onset of dystrophy* (days)
None	100	139
α-tocopherol acetate	0	
DPPD	62.5	235
Santoquin	87.5	182
DBH	100	231

* Data were based only on animals with dystrophy.

Findings indicate that DPPD, Santoquin and DBH delayed but did not prevent occurrence of dystrophy in guinea pigs fed a highly purified diet deficient in vit. E. In contrast to the results obtained with these antioxidants, alpha-tocopherol acetate was fully protective in this regard. Since the basal diet employed in these studies was free of vit. E and contained no cod-liver or other highly unsaturated oils, it would appear that the delay in onset of dystrophy on diets containing DPPD, Santoquin and DBH was due not to the sparing effect of these antioxidants on residual quantities of vit. E (which is readily destroyed in the presence of unsaturated fats, particularly fish oils) in the diet but rather to the sparing effect of these antioxidants on vit. E in the tissues. Findings suggest that as long as tissue stores of vit. E were adequate, dystrophy did not occur. Once tissue stores of this vitamin were depleted, however, the above antioxidants were ineffective in preventing dystrophy in contrast to the continued effectiveness of alpha-tocopherol acetate in this regard.

Summary. DPPD, Santoquin and DBH delayed but did not prevent the occurrence of muscular dystrophy in guinea pigs fed a highly purified diet deficient in vit. E. In contrast to these results, alpha-tocopherol ace-

tate was fully protective in this regard.

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Adaptation of ECHO Viruses in HeLa Cells; Their Use in Complement Fixation.* (23189)

Italo Archetti, Jean Weston, and Herbert A. Wenner
Department of Pediatrics and Hixon Memorial Laboratory, University of Kansas School of Medicine, Kansas City.

The adaptation of ECHO prototype viruses (1) or primary isolation of ECHO viruses in cultures of HeLa cells has not been reported previously. Twelve of the original 13 ECHO viruses have been propagated successfully in cultures of HeLa cells maintained in this laboratory. ECHO virus type 4 failed to grow in HeLa cells.

During preparation of ECHO hyperimmune sera in monkeys, difficulties were encountered in complement fixation (CF) tests with viral antigens derived from infected renal cells. Viral antigens derived from HeLa cells proved to be useful in delineating homotypic antibody, and in exposing serologic cross-relationships observed in neutralization tests. The present report describes the methods used in adaptation of ECHO viruses to HeLa cells, and the application of viral antigens derived from HeLa cells in the CF test.

Materials and methods. Viruses. The prototype ECHO viruses were furnished by J. L. Melnick, Wm. McD. Hammon and A. B. Sabin. We passaged the viruses once or twice in monkey renal epithelial cells(2) when a large pool of stock virus was prepared. The passage history and titers of viruses propagated in cultures of renal and HeLa cells appear in Table I. *Tissue cultures.* The line of HeLa cells was obtained from J. T. Syverton in 1952(3). Since 1955 the cell line has been propagated in yeast extract medium(4) modified as follows: 1% yeastolate (Difco), 8.0%; 20% glucose, 1%; Hank's BSS, 68.0%; 2.8% $NaHCO_3$, 3%; and human serum, 20.0%. Following satisfactory growth in tubes or in bottles the cells were washed 3 times with phosphate buffered saline (PBS). The maintenance solution used in this laboratory(5) consists of mixture 199, 63.5%; 5% $NaHCO_3$, 3%; 20% glucose, 1%; tryptose phosphate broth, 25%; and calf serum, 7.5%. Following addition of maintenance solution cells were ready for inoculation with ECHO prototype viruses. *Virus titrations.* The stock virus pools were titrated in roller tube cultures of monkey kidney epithelial cells. ECHO viruses adapted to HeLa cells were titrated in renal epithelial cell cultures in the colorimetric test originally described by Salk, Youngner, and Ward(6) as modified by Melnick and Opton(7). *Antisera.* Sera were obtained from monkeys (*Macaca mulatta*) hyperimmunized with 13 prototype ECHO

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TABLE I. Passage Histories, Cytopathogenic Intensities, Antigenic Potencies, and Virus Titers of ECHO Viruses in HeLa Cell Cultures.

ECHO virus	Passage history	Extent of cytopathogenic change in HeLa cultures (%)	Antigenic units, CF test	TCD_{50}/ml* virus derived from HeLa	TCD_{50}/ml* virus derived from Renal
Farouk	T_{10} K_9 H_5	90+	2	7.5	7.7
Cornelis	T_9 K_9 H_5	75+	1	6.6	5.8
Morrissey	T_5 K_{12} H_5	50+	1	6.6	7.5
Pesascek	T_9 K_6 H_5	±	1	<1.0	4.5
Noyce	K_8 H_5	75+	2	8.0	8.8
D'Amori	K_{11} H_5	90+	2	6.5	8.4
Wallace	K_6 H_5	90+	1	4.0	8.5
Bryson	K_7 H_5	75+	2	6.7	7.8
Hill	K_{10} H_5	75+	2	7.5	7.2
Lang	K_{13} H_5	75+	8	4.7	7.4
Gregory	K_8 H_5	90+	1	6.0	7.0
Travis	K– H_5	90+	1	6.8	8.8
Hamphill	K– H_5	90+	1	5.5	8.1

* Renal-cell-adapted viruses were titrated in renal cell roller tubes. HeLa-cell-adapted viruses were titrated in renal cell cultures in the color test based on decimal dilutions, expressed as log base $_{10}$.

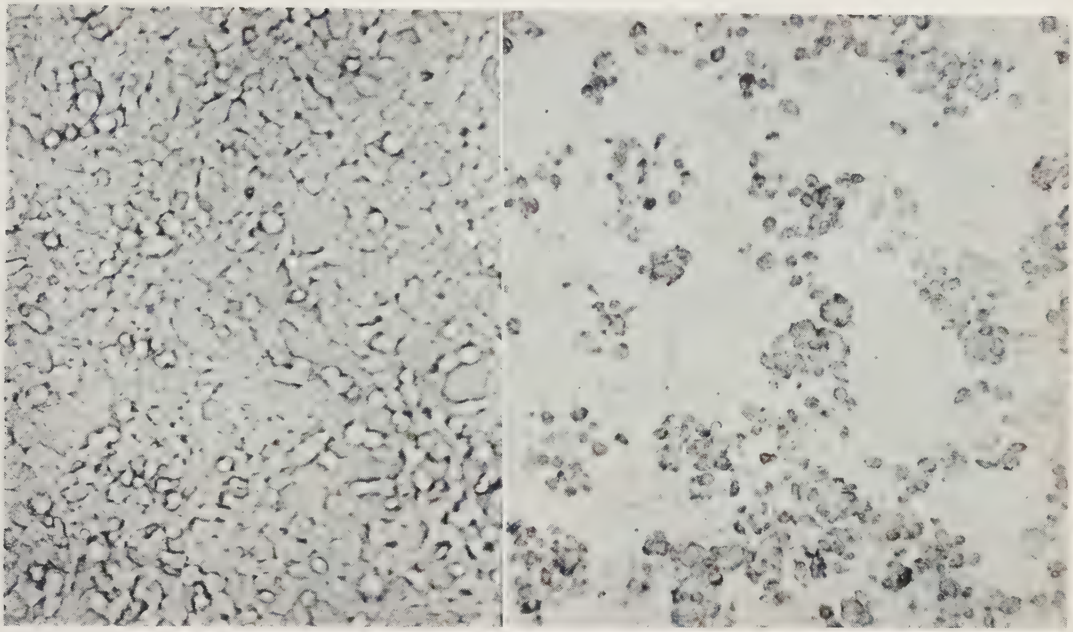
T_{10} K_9 H_5 = 10 passages in testicular fibroblasts, 9 additional passages in kidney cells, and 5 passages in HeLa cell cultures.

viruses.[†] Pre-vaccinal sera were devoid of homotypic and heterotypic neutralizing antibodies against the 13 ECHO viruses.[‡] The immunizing antigens were viruses propagated in monkey renal cells. Each monkey received a series of 5 or 6 intramuscular inoculations. Each inoculum consisted of 1.0 ml of undiluted virus suspended in 1.0 ml of mineral oil adjuvant(8). *CF antigens.* At the 4th passage level in HeLa cells cultured in stationary tubes, milk dilution bottles (200 ml) containing $10^{6.6}$ cells were inoculated with 0.5 ml of each of the 13 ECHO viruses. The volume of maintenance fluid overlaying inoculated cells was 8.0 ml. Cytopathogenic changes were well advanced with all, except type 4 virus, on the 5th day when fluids were harvested. The harvested fluids were treated immediately with $NaHCO_3$ in order to bring the pH to 7.4, and stored in glass-sealed vials at –20°C from whence they were deployed for use in CF tests. *CF tests.* The drop method described by Fulton and Dumbell(9) was adopted with several modifications, most of which have been described previously(10, 11). The concentration of sheep's red cells was 0.5%. The same lot of guinea pig complement was used in the study. Titrations of antibody were done using constant quantities of antigen and complement and doubling dilutions of serum. The unit of complement was taken as the least amount of guinea pig serum that caused complete lysis of the cells. Complement titers were verified by titrations performed coincidental with each test. Antigen, serum, hemolytic, and cell controls were employed. Known negative monkey sera were included in each test. Sera were inactivated at 60°C/30′. Primary incubation was carried out overnight (18 hours) at 4°C. The hemolytic system (1:1000 hemolysin plus 0.5% sheep's rbc) incubated 30 minutes at 37°C was added on the following morning. After a further period of incubation for 2 hours at 37°C the results were recorded. The antibody titer was the highest dilution of serum giving 50% inhibition.

Results. Cytopathogenic action of ECHO

[†] The following media were used in the propagation of viruses in monkey renal cell cultures which were used to hyperimmunize monkeys: *Growth medium.* Medium D = mixture 199, 95%; horse serum, 2%; and 2.8% $NaHCO_3$, 3%. The growth medium was usually replenished on the 3rd or 4th day. The cells were washed 3 times after which maintenance medium was added. *Maintenance medium* consisted of mixture 199, 97.5% and 5% $NaHCO_3$, 2.5%.

[‡] Unpublished data.



a. Normal HeLa cell culture b. Infected with ECHO 9

FIG. 1. Cytopathogenic action of ECHO 9 (Hill) in HeLa cells (unstained preparations 160×).

viruses on HeLa cells. Except for ECHO virus type 4, difficulty in adapting 12 ECHO viruses to cultures of HeLa cells was not encountered. In respect to type 4 cytopathogenic changes were doubtful and were not reproducible, although attempts were made in several serial passages. Furthermore at the 5th passage level measurable amounts of virus were not found on titration in monkey kidney epithelial cells (Table I).

Following primary inoculation of the remaining 12 ECHO viruses in HeLa cells, cellular changes were observed as early as the 3rd day and reached maximal degeneration on the 7th day when most of the cells were visibly affected. On the 3rd day post-inoculation the cells began to separate and had an irregularly rounded appearance. By the 5th or 6th day shrunken misshapen cells had arranged themselves in irregular clusters. Undoubtedly cellular lysis occurred, but the notable persistence of intact altered cells beyond the 7th day suggested that virus multiplication was not necessarily associated with cell destruction.

At each passage level, except the last, the harvested cells were rapidly frozen and thawed 3 times in dry ice and alcohol in order to rupture cells. Such treatment may have had an effect (controlled observations were not made) since in continuous passage there was evidence of adaptation in that fewer clusters of intact abnormal cells remained on glass or in fluid suspension by the 5th day post-inoculation. Despite the progressive effect on cellular lysis several passages by these means were not conducive to complete cellular destruction. The extent of cellular lysis ranged for the several viruses between 70 and 90%. The quantity of virus released in culture fluids ranged between $10^{4.0}$ and $10^{7.5}$ TCD_{50} (Table I). The type of change encountered with ECHO virus type 9 appears in Fig. 1. The changes shown are fairly characteristic of the observed cytopathogenic changes observed with other ECHO viruses propagated successfully in HeLa cell cultures.

Specificity of ECHO virus antigens in the CF test. Viral antigens derived from infected renal cells proved to be anticomplementary and cross-relations between members of the group were encountered in low serum dilutions, (*i.e.,* 1:32 or less). The anticomplementary effect of the antigens was not re-

TABLE II. Specificity of Hyperimmune ECHO Antisera Prepared in Monkeys. Results obtained in CF test.

ECHO virus antigen	Hyperimmune monkey antisera 1	2	3	4	5	6	7	8	9	10	11	12	13
Farouk	2048	8	0	0	0	0	0	32	0	0	0	32	128
Cornelis	8	1024	8	8	8	0	0	0	0	0	0	0	0
Morrissey	0	0	1024	0	0	0	8	0	0	0	0	0	0
Pesascek	—	—	—	—	—	—	—	—	—	—	—	—	—
Noyce	8	8	0	0	2048	0	8	0	0	8	0	0	0
D'Amori	8	0	0	0	0	2048	0	0	0	0	0	0	0
Wallace	0	0	0	0	0	0	256	0	0	0	0	0	0
Bryson	128	8	0	0	0	0	8	1024	0	0	0	16	32
Hill	8	8	0	8	0	0	8	8	512	8	0	8	8
Lang	16	0	0	0	0	0	0	0	0	1024	0	0	0
Gregory	0	0	8	0	0	0	0	0	0	0	512	0	0
Travis	16	8	16	0	8	0	0	0	0	0	0	64	0
Hamphill	1024	0	0	0	0	0	0	256	0	0	0	512	1024
Homologous serum neutralization endpoints* (× 1000)	16	16	25	.2 or <	25	32	16	12	16 or >	16 or >	4.1	20	9

* Against approximately 100 TCD_{50} of homologous virus.
0 = <1:8 dilution of serum.

moved by heat (56° to 62°C/30′). Moreover, the antigens prepared in renal cells were qualitatively poor in respect to complement-binding capacity. Antigens derived from HeLa cells on the other hand were almost devoid of anticomplementary effect and were qualitatively better, if not entirely ideal, in their application in the CF test.

The results reported here concern use of unheated antigens. Antigens prepared in the manner described had low titers ranging from 1 to 8 units, with an average of 2 units (Table I). Box titrations of antigens did not provide evidence of a zone phenomenon, indicating that minimal rather than excess amounts of antigen were used.

The CF antigens were specific and reliable. The homotypic titers of immune sera prepared in monkeys ranged between 1:64 (type 12) and 1:2048 (types 1, 5, and 6). The results of the tests appear in Table II. The specificity of the CF antibodies was confirmed in neutralization tests. The homotypic neutralizing antibodies are recorded in Table II.

Cross-relationships in the CF test were encountered largely with respect to 3 ECHO viruses, namely types 1, 8, and 13. Each of these viruses reacted also with type 12 antiserum. However, ECHO virus type 12, with the exception of a possible cross with type 1 antiserum, failed to react with type 8 and type 13 antisera. Minor one-directional relationships between ECHO type 10 virus and type 1 antiserum and ECHO type 12 virus and type 3 antiserum remain to be clarified. Except the minor one-directional relationships the results obtained in CF test reaffirmed previous observations on serologic relationships obtained in neutralization tests (Table III).

The cross-relationships encountered above suggested that ECHO viruses types 1, 8, 12, and 13 may share common antigens. However, interpretation on this point should be postponed until there is satisfactory evidence that serologic cross-relationships are reproducible using purified viruses. The chief point of interest concerns the specificity and the reciprocal relationships established in CF and in neutralization tests.

Discussion. The successful propagation of prototype ECHO viruses in HeLa cells was unexpected for the chief reason that primary detection of naturally occurring ECHO viruses in these cells has not come to our at-

TABLE III. Comparison of Cross-Antigenic Relationships Delineated by Neutralization and CF Tests with 4 ECHO Virus Hyperimmune Sera.

	Neutralization endpoints*				CF endpoints			
ECHO virus	1 Farouk	8 Bryson	12 Travis	13 Hamphill	1 Farouk	8 Bryson	12 Travis	13 Hamphill
Farouk	16,000	500	200	1024	2048	32	32	128
Bryson	1600	12,000	16	100	128	1024	16	32
Travis	<4	<4	20,000	<4	16	<8	64	<8
Hamphill	10,000	160	500	9000	1024	256	512	1024

* Approximately 50 TCD_{50} used; based on avg values of 4 titrations.

tention. Syverton[§] has not yet recovered ECHO viruses in HeLa cell cultures. Thus, it may be adduced that HeLa cells are less sensitive than renal cells for detection of ECHO viruses. This, however, is a relative matter, for strains of human origin may prove difficult to detect also in renal cells unless several passages are made. Maximal lytic effect may be observed only after several passages in cultures of renal cells(12). In respect to the adaptation to HeLa cells of virus stocks prepared in renal cells, two possibilities are suggested: namely, (a) that progeny delivered during passage in renal cells contain variants sufficiently represented in the total population to initiate attack of HeLa cells on a scale conducive to visualization, or (b) that the HeLa cell colony continuously in passage in this laboratory has undergone change in respect to susceptibility. The evidence in support of either proposition is insufficient at this time.

Infected fluids harvested from HeLa cells provided specific and reliable although relatively weak antigens in the CF test. ECHO virus type 10 was exceptional and provided a very satisfactory antigen. Heated ECHO viral antigens were less satisfactory than active viral antigens in 2 respects: namely, (a) loss of specificity as indicated by heterotypic cross-reactions with all antisera in low dilution (1:8), and (b) slightly lower homotypic serum dilution endpoints. Sufficient information has not been obtained to establish or to eliminate the presence of soluble antigen.

Better antigens undoubtedly can be prepared. Antigens prepared in cultures of renal cells may prove equal to, or superior to antigens prepared in HeLa cells for diagnoses of human illnesses caused by ECHO viruses. We turned perforce to HeLa cells because of the anti-complementary effect of antigens and antisera derived from monkeys. The cross-reactions observed with antigens derived from monkey renal cells and monkey hyperimmune sera are not entirely understood. The serologic evidence indicates that the cross-reactions were not the result of antibodies to any component in the growth or maintenance media, thereby suggesting interreaction in the monkey to some cellular component released along with virus in culture fluids used to immunize monkeys. Such relationships would not be observed in naturally occurring diseases in human beings or in animals. But whether antigens prepared either in HeLa or in renal cell cultures are sufficiently active for reliable use in measurements of CF antibody response in naturally occurring illness, where neutralizing antibodies remain at levels comparatively lower than in hyperimmunized monkeys, is a subject for additional study.

Summary. Twelve of 13 original ECHO viruses have been adapted to HeLa cells in tissue cultures. The adapted viruses produced discrete, recognizable cytopathogenic changes. Infected fluids at the 5th passage level provided specific, reliable antigens for use in the CF test.

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Enzymatic Release of Pantetheine from Coenzyme A in Animal Tissues And Its Microbiological Determination. (23190)

R. L. WOLFF, S. DUBOST AND J. J. BRIGNON (Introduced by Raymond Cahen)
Laboratoire de Chimie Biologique, Faculté de Médecine, Nancy, France.

Microbiological determination of pantothenic acid(1) or of pantetheine(2) released by enzymatic hydrolysis, has been recommended as a routine method for determination of coenzyme A in animal tissues. The first of these two methods requires the use of 2 enzymes, liver enzyme and intestinal phosphatase. With one enzyme, phosphatase, CoA is split into pantetheine. As the activity coefficient of pantetheine for growth of *Lactobacillus arabinosus* amounts to 20-40% (on a molecular basis) of that of pantothenic acid, microbiological determination of pantetheine, in the presence of preformed free pantothenic acid, becomes very difficult with *L. arabinosus*. With *Lactobacillus helveticus*, another difficulty arises owing to stimulation of the activity of pantetheine by small amounts of pantothenic acid present in the mixture.

Experiments are here reported pointing to a modification of the basal medium for *Lactobacillus arabinosus* 17-5 whereby pantothenic acid and pantetheine, in equimolecular amount, display an identical activity as growth factors. Each of the two factors is devoid of any stimulatory activity towards the other. This method has been checked with the double enzyme method of Novelli and Lipmann.

Method. The basal medium, for *L. arabinosus* 17-5, has been described by Skeggs and Wright(3). It was modified by addition of 50 ml of a 20 mg/ml solution of cysteine hydrochloride to 250 ml of the original medium and was adjusted to pH 6.6-6.8. The tubes were incubated in glass jars at 37°C, for 24 hours in an atmosphere of pure CO_2. *Substrates.* The Ca-pantothenate was of commercial origin. The pantetheine was used as its S-benzoyl derivative,* the identity of the activity for the 2 compounds having been demonstrated. It had been prepared according to Schwyzer(4). *Intestinal phosphatase* was prepared from calf intestinal mucosa according to the first steps of the method described by Schmidt and Thannhauser(5). The trypsin digested mucosa, after filtration, was dialyzed against tap-water for 72 hours and was kept in the freezer at –20°C. *Phosphatase activity* of the solution corresponded to 45 Thannhauser units per ml. Two ml of aqueous tissue extracts, prepared according to the procedure of Kaplan and Lipmann(6), were incubated for 3 hours at 37° with 0.4 ml phosphatase, 0.1 ml 0.12 M $NaHCO_3$ and 47.5 ml H_2O before the assay.

Results. The action of cysteine and CO_2 on growth of *L. arabinosus* in the presence of varying amounts of S-benzoylpantetheine or pantothenic acid is shown in Fig. 1 (I-IV). Cysteine, in the amount of 10 mg per tube of 5 ml, considerably increases activity of pantetheine but not that of pantothenic acid. In an atmosphere saturated with CO_2, activity of either growth factor is slightly increased but that of pantothenic acid to a lesser degree

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* Obtained through the courtesy of Dr. Schwyzer, Ciba Inc., Basel, Switzerland.

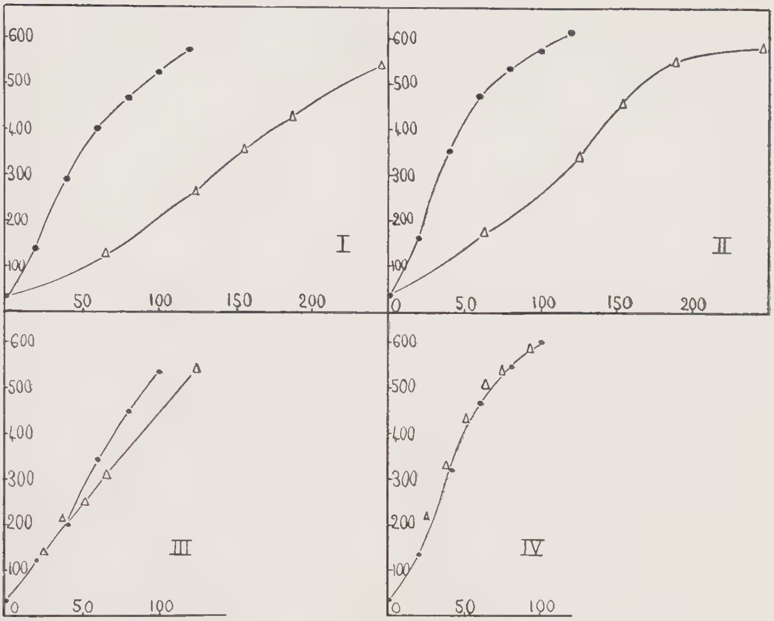

FIG. 1. Growth curves of *Lactobacillus arabinosus* in presence of S-benzoylpantetheine (△) or pantothenic acid (●). Influence of cysteine (10 mg/tube of 5 ml) and CO_2. Abscissa: Ca-pantothenate or S-benzoylpantetheine (on an equimolecular basis). Ordinate: Units (Meunier). I = Atmosphere: air; II = Atmosphere: CO_2 100%; III = Atmosphere: air + 10 mg cysteine per tube; IV = Atmosphere: CO_2 100% + 10 mg cysteine per tube.

than that of pantetheine. Under the combined influence of cysteine and CO_2, growth stimulation of *L. arabinosus* by pantetheine or pantothenic acid becomes identical. In contrast to *L. helveticus,* no potentiation occurs in the presence of the 2 growth factors, the total activity being (on a molecular basis) the sum of each individual activity.

Table I shows the results of microbiological determination of CoA in several tissues of rats, rabbits and guinea pigs. The two methods give identical results.

TABLE I. Determination of CoA Bound Pantetheine Released after Incubation of Tissue Extract with Intestinal Phosphatase and Calculated as Ca-pantothenate. (The number in the table corresponds to the sum: performed pantothenic acid + liberated pantetheine.)

Tissue	Liver		Heart		Kidney		Spleen	
Method	a	b	a	b	a	b	a	b
No. of rats	9	9	6	6	7	7	5	5
Mean	68.5	65.5	32.8	33.4	34.9	32.5	9.1	8.5
S.E. of mean	4.37	3.76	4.88	4.58	2.82	3.71	1.36	1.02

Tissue	Muscle		Brain		Testicles		Adrenals	
Method	a	b	a	b	a	b	a	b
No. of rats	2	2	3	3	2	2	1	1
Mean	9.8	11.5	8.5	8.6	13.1	13.1	26	30

Animal	Rabbit		Guinea pig	
Method	a	b	a	b
Liver	42.7	42.7	52.1	53
Kidney	41.8	40.8	29.1	27.8
Heart	21.5	19.4	10	9.4
Brain	10.7	11.1		
Adrenals			10.6	8.8

a = present method; b = method of Novelli and Lipmann.

According to Marnay(7), Cheldelin *et al.*(8) and Atkin *et al.*(9), heart and muscle contain considerable amounts of "conjugated pantothenic acid" of a different kind of CoA bound pantothenic acid. The conjugate is split by autolysis, by takadiastase, mylase P, etc. The amount of this "conjugated pantothenic acid" exceeds by far the amounts of CoA bound pantothenic acid as measured by the double enzyme method. It is interesting to note that the non-purified phosphatase used in these experiments is devoid of any "pantothenic conjugates" splitting activity other than CoA.

Summary. (1) A method has been described whereby pantetheine and free pantothenic acid may be determined microbiologically in a medium in which the 2 factors exert (on a molecular basis) the same effect on *Lactobacillus arabinosus*. The action of the 2 growth factors is additive. (2) The CoA bound pantothenic acid is liberated as pantetheine by incubation of an aqueous tissue extract with intestinal phosphatase and can be determined in the presence of preformed free pantothenate. (3) The results of this method, applied to several tissue extracts, agree with those of Novelli and Lipmann.

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Action of Protamine on Renal Potassium Excretion in the Rabbit. (23191)

ERNEST C. FOULKES,* IRWIN B. HANENSON,† AND BENJAMIN F. MILLER
May Institute for Medical Research, Jewish Hospital Assn., and Departments of Physiology and Medicine, University of Cincinnati College of Medicine.

Recent evidence has shown that protamine is an inhibitor of active potassium transport in kidney cortex slices of the rabbit(1). Sodium transport in that system is not affected. In this report, we describe some effects of protamine on renal electrolyte excretion in the rabbit. It was the purpose of these experiments to determine whether the effects of protamine in the whole animal parallel those observed in kidney slices from the same species.

Methods. Male albino rabbits weighing 7 to 8 lb were placed on a standard solid rabbit ration.‡ Prior to the experiment water or 0.45% saline was administered by stomach tube. Intravenous injections and infusions were given through a marginal ear vein. Urine was collected from an indwelling catheter by gentle suction with a syringe. The bladders of several animals sacrificed at the end of the experiment were examined for residual urine but were invariably found to be empty. Animals were restrained on their backs and blood collected from the heart at approximately the midpoint of each clearance period. The filtration rate was measured with creatinine administered by constant intravenous infusion or by subcutaneous injection one hour before the beginning of the experiment. Electrolyte determinations were carried out on a Baird flame photometer on suitable dilutions of serum and urine with lithium as internal standard. Creatinine was estimated as total chro-

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[*] Established Investigator, Amer. Heart Assn.

[†] Eda K. Loeb Memorial Scholar, May Institute.

[‡] Obtained from Rockland Farms, New City, N.Y.

TABLE I. Effect of Protamine on Potassium Reabsorption in Normal Rabbit (Animal H, 3.6 kg).

Elapsed time (min.)	Clearance period	Urine (ml/min.)	Creatinine clearance (ml/min.)	Sodium			Potassium		
				Plasma (mE/l)	Excretion (μE/min.)	% reabsorption	Plasma (mE/l)	Excretion (μE/min.)	% reabsorption
24	1	.54	19.3	136	1.3	99.9	4.50	10.8	87.6
44	2	.91	19.7	131	1.2	99.9	4.13	9.0	88.9
44-46	Inj. intrav. one ml isotonic protamine Cl								
60	3	1.58	19.5	132	2.6	99.9	4.45	21.2	75.6
86	4	.33	17.7	134	1.1	99.9	4.42	17.3	65.1
114	5	.46	20.8	138	1.4	99.9	4.30	10.0	88.8

Animal hydrated with 400 ml H_2O over period of 3 hr before beginning of exp. Creatinine 15 ml 5% subcut. one hr before beginning of exp.

TABLE II. Effect of Protamine on Potassium Excretion in K-Loaded Rabbit (Animal L3, 3.6 kg).

Elapsed time (min.)	Clearance period	Urine (ml/min.)	Creatinine clearance (ml/min.)	Potassium		Ratio $\frac{\text{Excretion}}{\text{Filtered}}$
				Plasma (mE/l)	Excretion (μE/min.)	
0	Infuse 3% KCl at 0.6 ml/min.					
7	15 ml 5% creatinine subcut.					
59- 88	1	.94	20.4	7.4	183	1.21
103-105	Inj. 1 ml isotonic protamine Cl					
123	2	.80	24.9	6.6	201	1.22
135	3	.50	23.2	6.8	168	1.06
148	4	.35	23.6	7.2	139	.82
163	5	.40	23.7	7.6	156	.87
178	6	.37	21.0	8.4	146	.83

Animal hydrated with 300 ml 0.45% NaCl over period of 90 min. before beginning of infusion.

mogen by a modification of the method of Folin and Wu(2). For the potassium loading experiments animals were given 100 ml of 3% KCl daily by stomach tube for one week before the experiment. Potassium was infused at a constant rate throughout the experiment. Protamine was used as an isotonic solution of protamine chloride containing 262 micromoles chloride and 45 mg protamine per ml. Protamine chloride was prepared from protamine sulfate[§] by treatment with $BaCl_2$.

Results. Five animals were studied without potassium loading. In 4 animals, protamine caused a very marked decrease in percent potassium reabsorption. The values fell from 90.1 ± 2.6% (range 88.3 to 94.0) to 67.3 ± 5.4% (range 64.3 to 75.5). One animal showed no significant effect. Table I presents the results of one experiment.

Five potassium loaded animals were similarly treated with protamine. In 4 animals the potassium excreted initially exceeded the filtered load. In each case there was a significant drop in the ratio of K excreted/K filtered within 20 to 30 minutes after injection of protamine. The ratios of K excreted/K filtered were depressed by protamine from 1.21 to 0.83; 1.35 to 1.05; 1.08 to 0.95; 0.96 to 0.62; and 1.33 to 1.16; respectively. An experimental protocol is shown in Table II.

Discussion. Protamine strongly inhibits active accumulation of potassium by kidney slices(1). The present experiments have revealed a close parallel between the actions of protamine on potassium transport *in vivo* and *in vitro.*

In the intact animal the kidney can transport potassium both into and out of the tubules(3,4). Injection of protamine increased the ratio of potassium to creatinine clearance in non-loaded animals. This indicates inhibition of potassium reabsorption. Indeed, if protamine under these conditions primarily inhibited K secretion, a decreased potassium clearance would have been observed. In the potassium loaded animals the ratio of potassium to creatinine clearance was significantly depressed on administration of protamine. Such a finding is explained by inhibition of tubular potassium secretion.

[§] Obtained from spring salmon and purchased from General Biochemicals, Chagrin Falls, O. This product conforms to the standards of the Federal Register for preparation of protamine-zinc-insulin.

In animals to whom no NaCl had been administered, there was no effect of protamine on sodium transport. This observation agrees with previous findings on rabbit kidney *in vitro*. Animals hydrated with hypotonic saline showed a relatively high control Na excretion which fell throughout the experiment as the administered sodium load was excreted. The absence of a protamine effect on sodium reabsorption supports the conclusion of the previous *in vitro* studies that protamine is a specific inhibitor rather than a general cytotoxic agent.

Conclusions. Some effects of intravenous administration of isotonic solutions of protamine chloride on renal electrolyte clearance in the rabbit were studied. In this species protamine inhibits both potassium reabsorption and potassium secretion. There were no significant effects on sodium clearance.

It is a pleasure to thank Miss B. J. McCardwell and Mr. L. Staples for their help.

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Effects of Essential Fatty Acid Deficiency in Young Swine.* (23192)

Eldon G. Hill, Egis L. Warmanen, Herbert Hayes, and Ralph T. Holman
Hormel Institute, Departments of Animal Husbandry and Physiological Chemistry, University of Minnesota, Austin.

Since Burr and Burr(1) demonstrated the need of rats for essential fatty acids (EFA), these substances have been found to be required by several species ranging from insects to ruminant mammals(2). Witz and Beeson (3) found that the effects of a low-fat diet upon weanling pigs were loss of hair, scaly dermatitis, and necrotic areas on the skin. They did not specifically investigate the need of swine for EFA. Studies of EFA deficiency in large animals have been particularly difficult because of inability to deplete the weanling animal of its stores of EFA within a reasonable length of time. With development of a successful procedure for raising baby pigs without colostrum on a purified diet, and availability of baby pigs obtained by hysterectomy(4) from dams of the Hormel Foundation herd of miniature swine(5), it became feasible to attempt a study of essential fatty acid deficiency in swine. An attempt was also made to accelerate EFA-deficiency by feeding of cholesterol, as was recently done with rats(6).

Materials and methods. Four trials were conducted using a total of 15 miniature pigs in the first 3 experiments and 7 Chester White pigs in the fourth experiment. All pigs were obtained by hysterectomy 3 to 6 days before expected parturition. The pigs were held in individual isolation cages for 4 weeks and then transferred to larger individual open wire mesh cages, with wire mesh floors. The pigs were fed a pre-experiment diet consisting of whole cows' milk supplemented with one whole egg, 5 ml of mineral mixture,† 400 IU vit. D_2, 30 mg chlortetracycline, and 1 mg of

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[†] Each liter of mineral mix contained 50 g $FeSO_4 \cdot 7H_2O$, 4 g $CuSO_4 \cdot 5H_2O$, 4 g $MnCl_2 \cdot 4H_2O$, 0.30 g KI, 0.30 g $ZnCl_2$, 0.25 g $CoSO_4 \cdot 7H_2O$ and 4 ml HCl.

TABLE I. Growth and Feed Consumption of Young Swine Fed Purified Diets Low in Essential Fatty Acids, and Effect of Supplementation with Ethyl Linoleate.

Exp. and pig No.	Linoleate supplement	Days on exp.		Body wt, lb Start	End	Intake of polyunsat., g	Lb feed per lb gain	Aortic lesions
I 165-5	—	89	S	5.4	9.5	2.10	9.6	—
191-2	—	45		3.1	3.0	1.10		—
	+	5	D	3.0	3.0	5.11		
191-1	—	69		3.7	4.7	1.57	30.4	—
	+	27	S	4.7	8.7	27.92	4.5	
II 340-3	—	45	D	2.4	13.2	1.71	3.0	—
341-4	—	83	S	3.1	42.0	7.34	3.6	+
340-4	—	84	S	2.6	41.0	6.01	3.0	—
340-10	—	84	S	2.5	37.0	5.67	3.2	—
341-5	+	83	S	3.1	28.0	87.09	3.2	—
340-2	+	84	S	2.7	35.0	90.40	3.8	—
III 369-3	—	31	S	2.0	5.0	.67	3.6	—
369-2	—	43	D	1.7	5.6	1.02	5.1	—
369-9	—	44	S	2.0	8.2	1.25	4.0	—
369-1	—	46	D	1.6	8.5	1.19	3.4	—
369-5	—	48	D	2.0	8.5	1.47	4.4	—
369-4	—	91	S	2.1	33.8	5.35	3.3	+
IV 3105-5	—	9	S	2.5	2.6	.14		—
3105-7	—	14	S	3.1	2.1	.21		—
3104-2	—	34	D	3.8	8.7	1.05	4.2	—
3106-5	—	48	D	3.6	9.7	1.54	4.9	—
3106-2	—	95	D	3.4	30.5	6.30	4.5	+
3106-1	—	98	S	3.8	39.0	7.29	4.0	+
3106-6	—	98	S	3.6	38.0	6.81	3.9	+

S—sacrificed; D—died.

vit. K per quart of homogenized milk. The first experiment was started when the baby pigs were 20 days (1 pig) and 7 days (2 pigs) of age. The remaining 3 experiments were started when the pigs were 11 days of age or less. Two basal diets were used in the experiments. Each kilogram of diet S5 contained 552 g glucose, 200 g vitamin-free casein, 80 g gelatin, 50 g hydrogenated coconut oil, 50 g cellulose (Alphacell), 3 g DL-methionine, 200 mg chlortetracycline, 5 g cholesterol, 48 g minerals,[‡] and vitamins[§] well in excess of NRC recommended allowances. The S7 diet differed from S5 in that it contained 1 g methionine, 200 mg $ZnSO_4 \cdot 2H_2O$, 0.5 mg biotin, 55 mg calcium pantothenate, and 2 mg vit. K per kg. Diets were mixed semi-weekly and stored at –20°C. The diets were fed as dry meal rations and acceptability was good in most cases. Food was given *ad libitum* until daily consumption reached 1 kg, the maximum allowed each animal. The total lipid content of each of the 2 rations extractable by ethyl ether was 6.4%. Analysis of the extractable lipid of diet S7 for polyunsaturated acids(7) revealed the presence of 0.14% dienoic acid, 0.05% trienoic acid, 0.02% tetraenoic acid, 0.03% pentaenoic acid, and 0.05% hexaenoic acid. Diet S7 provided 260 mg of total polyunsaturated acids/kg and diet S5 contained 114 mg/kg. Ethyl linoleate used for supplementation had an iodine value of 163, contained a trace of *trans* unsaturation as measured by infrared analysis, and 0.1% conjugated dienoic acids. The pigs were weighed weekly and the physical condition of the animals was observed. When pigs died during the experiment or were sacrificed at the end of the experiment, the organs were inspected for abnormalities and samples of hearts and livers were taken for

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[‡] Minerals added/kilo diet were 35 g $CaHPO_4$, 5 g NaCl, 6 g K_2HPO_4, 1 g $FeSO_4 \cdot 7H_2O$, 400 mg $MgSO_4 \cdot 7H_2O$, 250 mg $MnSO_4 \cdot 4H_2O$, 40 mg KI, 40 mg $CuSO_4 \cdot 5H_2O$, 20 mg $CoSO_4 \cdot 7H_2O$, 100 mg $ZnSO_4 \cdot H_2O$.

[§] Vitamins added/kg diet were 2 g choline chloride, 0.2 mg biotin, 11 mg thiamine · HCl, 22 mg riboflavin, 26 mg calcium pantothenate, 22 mg pyridoxine · HCl, 110 mg nicotinic acid, 2 mg folic acid, 1 mg vit. K, 110 μg vit. B_{12}, 9090 IU vit. A acetate, 909 IU vit. D_2, and 100 mg alpha tocopherol acetate.

TABLE II. Composition of Heart Fat and Liver Fat of Young Swine.

			Fatty acids, % of heart fat			Fatty acids, % of liver fat		
Exp. No.	Linoleate supplement	Days on exp.	Tetraenoic	Trienoic	Dienoic	Tetraenoic	Trienoic	Dienoic
I	—	89	3.9	3.1	13.8	3.0	2.1	2.0
	—	45						
	+	5	3.7	1.7	11.1	7.5	2.4	9.2
	—	69						
	+	27	1.7	1.9	4.7	4.9	5.6	6.9
II	—	45	1.9	3.1	1.7	1.4	4.9	.6
	—	83	2.0	7.8	3.1	1.6	6.4	2.1
	—	84	1.4	5.2	2.2	1.3	6.1	3.2
	—	84	1.4	6.0	2.5	1.7	7.0	3.0
	+	83	3.9	5.1	9.3	1.5	6.2	2.8
	+	84	3.6	3.7	9.2	4.5	4.6	4.0
III	—	31	3.7	5.5	5.7	3.0	9.4	3.3
	—	43	1.9	5.9	3.5	3.4	11.7	2.7
	—	44	2.5	8.0	4.4	2.3	8.1	1.8
	—	46	1.9	9.3	2.1	2.5	8.3	.9
	—	48	2.3	8.4	4.5	.8	5.4	1.0
	—	91	.0	.2	.0	4.2	4.6	2.5
IV	—	9	8.4	1.8	12.9	7.9	3.1	5.7
	—	14	7.4	1.1	8.1	15.8	4.5	4.4
	—	34	2.5	3.4	3.8	1.4	.8	1.8
	—	48	2.7	5.9	4.8	2.1	6.7	3.3
	—	95	2.7	7.4	4.9	.6	1.8	.4
	—	98	1.9	6.0	1.6	1.4	4.4	.5
	—	98	1.4	3.3	1.5	1.7	5.2	.6

analysis.

Results. Table I indicates the general plan and results of the experiment, and Table II gives the contents of polyunsaturated acids in the heart and liver lipids of all the pigs studied.

Exp. 1. Three miniature pigs were used in this trial. Pig 165-5 was started on experiment at 20 days of age, reached a maximum weight of 10.5 lb at 9 wk after which the weight declined slowly to 9.5 lb. The animal became listless, and developed a very rough hair coat but no skin lesions were observed. It was sacrificed when its condition became poor. The 2 other pigs in this experiment were started at 7 days of age, grew poorly for 3 wk, and subsequently declined in weight. Pig 191-2 was given 5 g of ethyl linoleate in 2 oral doses beginning the 49th day of the experiment, but was in such poor condition that it died 5 days later. Pig 191-1 was given 1 g ethyl linoleate daily for 27 days after 69 days on the EFA-low diet. This pig gained 4 lb in 27 days, the hair coat became smooth and the animal gained in vigor and improved in appetite. The supplementation resulted in a drop from 30 lb feed required per lb gain to 4.5 lb feed required per lb gain. The experiment was stopped at 27 days because the pig grew too large for its cage.

Exp. 2. Six miniature pigs were started on the experiment at 11 days of age. All were fed the depletion ration S5 and 2 were supplemented from the beginning with 1 g ethyl linoleate daily by mouth. Pig 340-3 died after 45 days on experiment. It had a rough hair coat but no skin lesions. The remaining 5 pigs were killed at 83 or 84 days on experiment. Post mortem examination revealed no striking differences except that the aorta from pig 341-4 had extensive rough areas (Fig. 1).

Exp. 3. Six miniature pigs 2 days of age were started on ration S7 with the intention that after 8 wk some pigs would be supplemented with ethyl linoleate. Pigs 369-3 and 369-9 were lost because of accidental injury, and 3 pigs died abruptly at 43, 46, and 48 days. Postmortem examination revealed no gross abnormalities. The sixth pig survived 91 days, and postmortem examination revealed mild gross lesions of the aorta similar to those shown in Fig. 1.

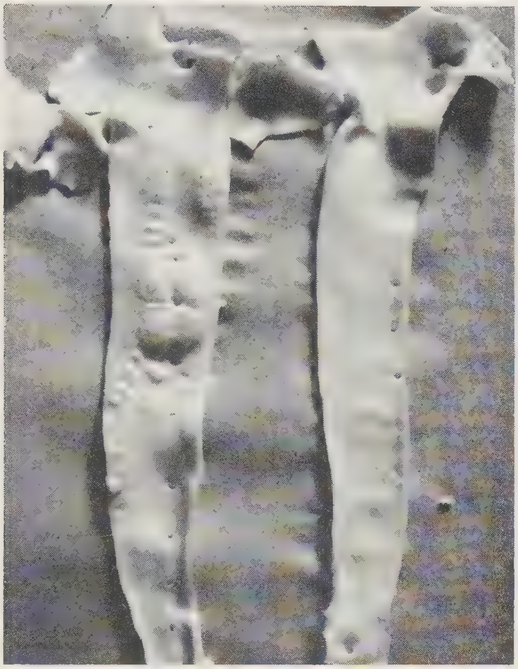

FIG. 1. Gross aortic lesions from pig 341-4 left, compared with littermate 341-5.

Exp. 4. Seven Chester White pigs were fed ration S7. Four of these pigs were started at 5 days of age and 3 at 1 day of age. Four pigs of the group either died early or had to be sacrificed because of impending death, leaving 3 which remained on the experimental regime more than 56 days. Of the 3 remaining pigs, 2 developed lameness in the rear legs and one of the lame pigs (3106-2) died abruptly at 95 days. This pig developed pin point skin lesions over the entire body, especially on the ears, over the back, and on the ventral surface. The remaining 2 pigs were killed at 98 days on experiment. Postmortem examination revealed very rough areas in the aorta in all 3 of the pigs that lived over 56 days.

Discussion. The original intent of the experiments was to attempt to produce dermal symptoms of essential fatty acid deficiency in the pig or to discover what form such deficiency takes in this species. Dermal lesions were observed in only one pig. The deficient diet caused poor growth and listlessness, and it was possible to maintain only a few of the pigs for any length of time on the diet. However, it became apparent that the diet caused an essential fatty acid deficiency because of the lowered polyunsaturated acid content of the tissues analyzed.

In Table II the analyses for polyunsaturated acids in the lipids of the hearts and livers are listed. The pigs are grouped by experiment and by length of time they were on the experimental diet. In general, the longer the animal was on the low EFA diet, the lower was the content of dienoic and tetraenoic acids in its heart and liver lipids. The content of these acids in the lipids was increased by supplementation with linoleic acid. This is as would be expected, for linoleic acid is known to be converted to arachidonic acid in the animal(2). The contents of pentaenoic and hexaenoic acids were measured, but are not presented because they were quite low and variable. The content of trienoic acids is likewise variable and shows no distinct upward trend as might be expected from experiments with other species(2). The contents of dienoic and tetraenoic acids of the liver and heart lipids show some variability which may be due to the differences in physical condition of the animals prior to death or sacrifice. Nevertheless, the trend toward lowered polyunsaturated acids in tissues indicates that an essential fatty acid deficiency was induced to varying degree in the experiments.

After this series of experiments was started, it became apparent that essential fatty acids are in some way related to cholesterol transport(6) and hence possibly to the general

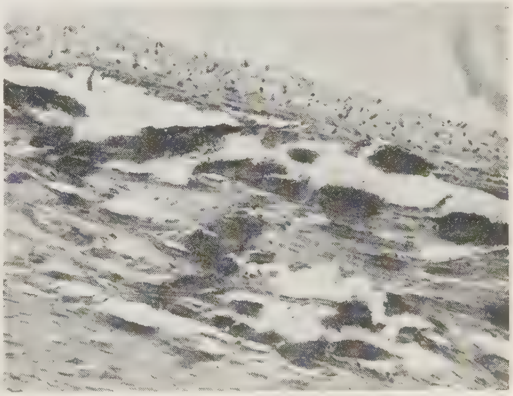

FIG. 2. Photomicrograph of lesion of aorta from pig 341-4. Hematoxylin-eosin stain. 115 ×.

problem of atherosclerosis. Therefore the aortas of the pigs fed the diets deficient in essential fatty acids were inspected for lesions. Of the 8 pigs that survived longer than the normal weanling age of pigs, 56 days, 5 were found to have gross lesions of the aorta. None of the 4 pigs supplemented with ethyl linoleate were found to have gross lesions. Microscopic examination of the lesions by Dr. Jesse Edwards of the Mayo Foundation confirmed the macroscopic observations. The lesions were found to be in the inner third of the media, and consisted of areas of calcification. No lipid deposits were demonstrable by Sudan IV staining. Alizarin red and von Kossa stains for calcium were positive, and Prussian blue stain for iron was positive. Ceroid stain was negative.

Summary. Rigid exclusion of essential fatty acids from the diet of young swine leads to a deficiency disease not characterized by consistent or early skin lesions. The severity of the dietary regime imposed caused high mortality, but of the 8 pigs that survived beyond the normal weanling age, skin lesions were observed in one and aortic lesions in 5, at or before 98 days on experiment. The data suggest that essential fatty acids are required by swine and that deficiency of essential fatty acids may lead to impairment of the arterial wall.

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Effect of Time and Temperature upon Survival of *Escherichia coli* in Sodium Chloride.* (23193)

HENRY C. REEVES AND ARTHUR P. HARRISON, JR. (Introduced by Charles C. Randall)
Department of Biology, Vanderbilt University, Nashville, Tenn.

When cells of *Escherichia coli* are suspended in 4.6 M NaCl and held at –22°C, the decline in number of viable cells does not proceed at a constant rate but at a continuously decreasing rate(1). To gain an insight into the kinetics of this decline, the effect of time and temperature upon survival has been investigated. The present paper summarizes some of the results of this investigation.

Materials and methods. The test organism was *Escherichia coli* strain 69L-15. It was cultivated in broth of the following percentage composition: yeast extract (Balt. Biol. Lab., dehydrated), 1.0; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.01; and "Tween 80" (Atlas Powder Co.), 0.1. The plating medium was of the same composition as the broth except 1.6% agar was an additional ingredient. Incubation was at 37°C. Cells of the test organism were transferred with a wire loop from a refrigerated stock agar culture to 5 ml of broth in a test tube and incubated for 24 hours. After 2 or more serial transfers through such tubes, 0.1 ml of the culture was used to inoculate 100 ml of broth in a 250 ml Erlenmeyer flask. After incubation for 24 hours, 30 ml of culture were centrifuged at 2,000 G for 30 minutes; the cells were washed twice with distilled water and finally resuspended to a volume of 30 ml with distilled water. The resulting aqueous suspension was

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immediately placed in an ice-water bath at +2°C.

One part of the aqueous bacterial suspension was mixed with 9 parts of 5.1 M NaCl at +2°C in the ice-water bath. This resulted in a 10-fold dilution of the cells and a reduction in concentration of the NaCl to 4.6 M. The pH of the mixture was 6. The bacteria-saline mixture was then dispensed in 5 ml aliquots into test tubes (15 mm x 150 mm) fitted loosely with aluminum caps. These manipulations at +2°C were completed in less than 3 min; then the tubes were placed at various temperatures for storage. The viable cell count at the moment of admixture of the bacteria with the saline was determined by a plate count on the original aqueous bacterial suspension and dividing this count by 10. This will be called the initial count. Four storage temperatures were employed: –22°, –9°, +2°, and +15°C. The tubed suspensions were maintained at –22° and –9°C in glycerol baths and at +2° and +15°C in water baths. When, during the course of an experiment, it was desired to alter the storage temperature the suspension was simply transferred from one bath to another. Only a few minutes were required for the suspension to attain the new temperature. Temperature was monitored by means of toluene thermometers and also by means of a "TRI-R" electronic thermometer (TRI-R Instruments, Long Island City, N. Y.), the sterile thermistor probe being placed within one of the tubed suspensions. The 4.6 M NaCl prevents solidification at –9° or –22°C, hence the effect of temperature may be studied in the absence of ice formation. To determine the number of survivors after storage in 4.6 M NaCl, 0.1 ml aliquots were withdrawn periodically and diluted serially in bottles containing 100 ml of diluent. Pour plates were prepared by plating 0.1 or 1. ml aliquots from these bottles. The plates were incubated for 24 hours. The diluent employed in the dilution bottles consisted of 0.05% trypticase (Balt. Biol. Lab., dehydrated) adjusted to pH 7.4 with phosphate buffer.

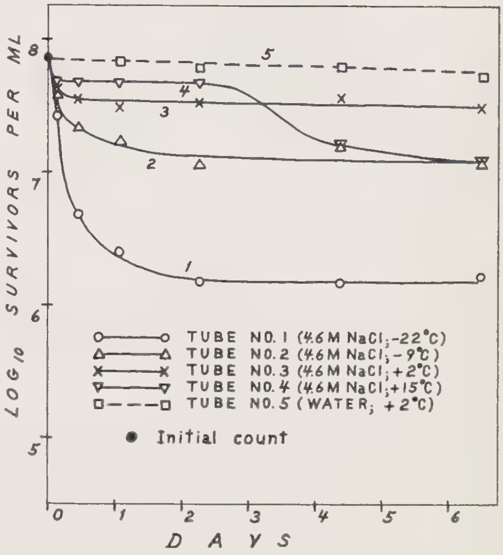

FIG. 1. Effect of temperature upon survival of *Escherichia coli.*

Results. Fig. 1 compares survival of *Escherichia coli* in 4.6 M NaCl at 4 temperatures (curves 1, 2, 3, and 4). It will be noted that survival at –9°C is better than at –22°C; similarly, survival at +2°C is better than –9°C. The relationship between survival at these 3 temperatures (curves 1, 2, and 3) is maintained throughout the 6½ day period. For the first 2 days, survival at +15°C is as might be predicted from the results at –22°, –9°, and +2°C. However, sometime after 2 days the death rate increases, then diminishes, and the curve "levels off" once again (curve 4). Between –22°C and +2°C survival is better the higher the storage temperature. Above +2°C, however, the configuration of the survival curve may vary from one experiment to another. In some experiments, the viable count remains higher at +15°C than at any of the other temperatures over the entire test period (1 week and longer). In other experiments, the curve is sigmoidal (Fig. 1). Since this behavior above +2°C occurs despite our uniform experimental technic, we have assumed that it is due to inherent differences between different cultures.

Fig. 2 illustrates that holding *E. coli* at +2°C for various periods prior to storage at –22°C will improve subsequent survival at the latter temperature. Holding the cells at +2°C for as short an interval as 3 hours improves survival at –22°C considerably (compare curve 2 with curve 1). Even after the

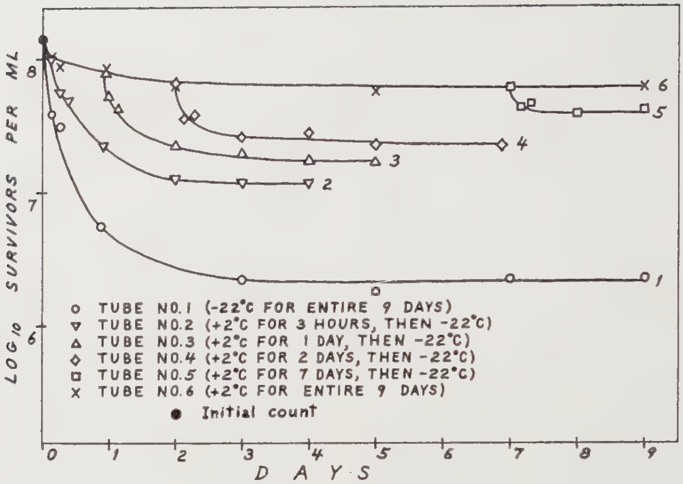

FIG. 2. Effect of length of holding at +2°C upon subsequent survival of *Escherichia coli* at -22°C.

suspensions at +2°C have become static, that is, after the death rate has become negligible (2 days, curve 6), the cells are undergoing some change, because continued holding at +2°C improves subsequent survival at -22°C even more (compare curve 4 with curve 5). In some experiments prior storage at +2°C for as little as 4 days conferred to the suspension complete resistance to -22°C. Improvement in survival at -22°C may also be acquired by prior storage at -9°C.

Fig. 3 summarizes data obtained from fluctuating *E. coli* suspensions between -22° and +2°C. If, during the period of rapid decline at -22°C, the suspension is transferred to +2°C, death will be arrested. In other words, during the period of rapid decline at -22°C, degree of death varies in proportion to length of exposure to this low temperature (compare curves 2, 3, and 4). Once the death rate of the suspension has diminished to a negligible value (after 1 day) warming to +2°C brings about no demonstrable change in viable cell count. In fact, once a suspension has attained this relatively static condition, fluctuations between -22° and +2°C have no effect whatsoever upon survival (compare curves 4 and 5).

The cells which remain viable during the period of rapid decline are able to maintain themselves for long periods. This resistance,

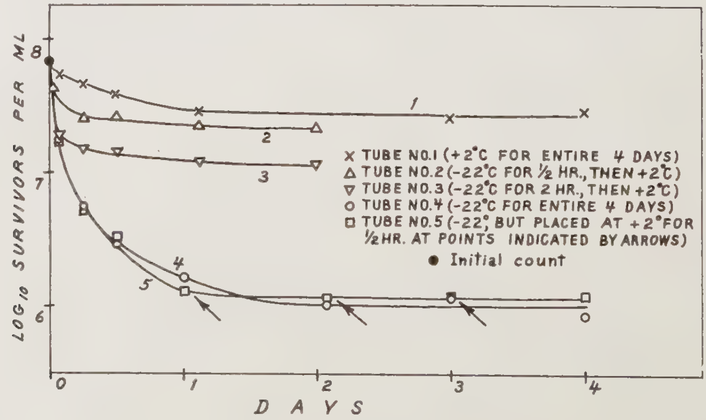

FIG. 3. Effect of temperature fluctuations between -22°C and +2°C upon survival of *Escherichia coli.*

however, is not heritable. If an aliquot of a stored (–22°C for 1 week) suspension is plated and the resulting colonies emulsified with water and subjected to a second exposure to 4.6 M NaCl, they are found to be no more resistant than an emulsion of colonies prepared from the original aqueous suspension.

The test organism will not grow at 37°C in broth containing 1.1 M NaCl. Therefore, it would hardly be expected to proliferate in a concentration of salt over 4 times as great in the absence of nutriments and at temperatures between +2° and –22°C. Even in the absence of salt there is no increase in the viable cell count at +2°C (curve 5; Fig. 1). In fact, there is no increase in viable count after 2 weeks in broth at this temperature. Nevertheless, it was considered prudent to make microscopic counts on the cell suspensions. No change in the number of cells per unit volume was detected from zero time to 1 week at either +2° or –22°C, nor was the number different at these 2 temperatures after any given storage period. Suspensions which had been fluctuated between +2° and –22°C showed no change in cell numbers. (The microscopic counts were made with dark field illumination, since the similarity in refractive index between the 4.6 M NaCl and the cells rendered detection of the latter difficult otherwise.)

Discussion. Suspensions of *E. coli* in 4.6 M NaCl stored at low temperatures undergo at first a rapid decline in viable cells, but as the storage interval is extended the death rate continuously decreases until eventually it becomes negligible. Cells that endure the period of rapid decline are able to maintain themselves in the saline for long periods. Most important, however, is the fact that once these cells are removed from the saline their resistance to it is lost. One may demonstrate this by centrifuging the cells from the NaCl and resuspending them in diluent. Upon a second exposure to 4.6 M NaCl, the suspension will undergo a decline proportionally as great as that obtained upon the first exposure. This same result is obtained with *Lactobacillus fermenti*(1). These data suggest that the resistance is acquired while the cells reside in the saline and does not exist preformed in a segment of the original population. Additional support for this idea is the adjustment which occurs when a suspension is held at +2°C prior to subsequent storage at the more lethal condition of –22°C. Here all cells (that is, all those which have not already succumbed at +2°C) may acquire resistance to salt at –22°C.

The above discussion leads to the consideration that the cells may adapt to the adverse environment presented by 4.6 M NaCl. Those cells which, by chance, miss the lethal event for a sufficient period have time to make the adjustments necessary to enable them to resist the event when it does occur. Such activity by the cells would account for the effect of temperature (Fig. 1), the improvement acquired by holding at a relatively high temperature prior to subsequent storage at a low temperature (Fig. 2), and also the continuously decreasing slope of the survival curve at a given temperature. Both death and adjustment could presumably result from the same stimulus. Which occurs would depend upon the conditions within the cell that influence the rates of the 2 processes. This hypothesis, therefore, assigns to the cell an active role in adjustment. One alternate hypothesis must be considered. The adjustment to NaCl tolerance may be the result of an effect brought about by the NaCl alone, for example, some diffusion phenomenon—possibly dehydration. According to this hypothesis the cell would have no active role in adjustment. The first of the 2 hypotheses given above was formulated by Eddy and Hinshelwood(2,3,4) to account for the behavior of declining populations of *Aerobacter aerogenes* at +25°C in proflavine, *m*-cresol, and low pH.

E. coli strain 69L-15 attains the "maximum stationary phase" in 8 hours or less. The cells used in our experiments are from cultures 24 hours old. Therefore, "cold shock," which occurs only with cells in the "logarithmic phase"(5), would not be expected to be a factor here. This is substantiated in our experiments. When a suspension is transferred from +2° to –22°C it cools to the latter temperature within a very few minutes. In spite of this, death continues for hours, unless it is arrested by placing the suspension back at +2°C (compare curves 2, 3, and 4; Fig. 3). Furthermore, relatively little death results upon exposure to –22°C in distilled water. In one experiment the initial viable cell count was 1.4 x 10^8 per ml; after 1 day the viable count was 1.2 x 10^8 per ml.

Since the NaCl in the suspensions prevents freezing, we are not concerned with extracellular ice. However, the possible formation and role of intracellular ice must be considered. Although our experiments do not permit a definite conclusion as to the presence or absence of intracellular ice, they do demonstrate that, if this ice is formed, it is not an important lethal factor. Firstly, periodic fluctuations from –22° to +2°C and back to –22°C bring about no greater death than uninterrupted storage at –22°C (curves 4 and 5; Fig. 3). Secondly, we have never detected between +2° and –22°C a crucial temperature on each side of which survival differs sharply.

Summary. Suspensions of *E. coli* in 4.6 M NaCl stored under conditions which preclude cell division undergo at first a rapid decline in viable cells, but as the storage interval is extended the death rate continuously decreases until eventually it becomes negligible. Cells that endure the period of rapid decline are able to maintain themselves in the saline for long periods. This resistance is not heritable. In fact, once the cells are removed from the saline their resistance to it is lost. Generally, the higher the storage temperature the better is survival. Also, holding suspensions at a relatively high temperature (+2° or –9°C) prior to subsequent storage at a low temperature (–22°C) improves survival at the low temperature. These data suggest that the tolerance to 4.6 M NaCl is acquired while the cells reside in the saline and does not exist preformed in a segment of the original population. Two hypotheses have been offered to account for the adjustment to NaCl tolerance.

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Swine and Experimental Atherosclerosis. (23194)

J. H. BRAGDON, J. H. ZELLER, AND J. W. STEVENSON (Introduced by J. H. Baxter)
Laboratory of Cellular Physiology and Metabolism, Nat. Heart Inst., Bethesda, and the Swine Research Section, Agric. Research Service, Beltsville, Md.

Gottlieb and Lalich(1) have reported that the aortas of swine obtained from slaughterhouses occasionally show atheroma-like lesions, and that incidence of these lesions increases with age. Lewis and Page(2) have reported rather high serum cholesterol levels and rather high concentrations of low-density lipoproteins in at least one strain of pig. It was therefore hoped that this species might prove a suitable one for studies on effects of diet on serum lipoproteins and on the atherosclerotic process.

Materials and methods. The experimental animals were 8 2-year-old boars of a crossbred strain, bred and maintained at Beltsville. They had previously been used in breeding experiments. Their basal diet consisted of a mash made of 60 parts yellow corn meal, 20 parts soybean oil meal, and 2 parts of mineral mixture. This diet contains approximately 2.9% crude fiber, 58.3% nitrogen-free extract, 17.6% protein, 3.2% fat, 4.8% ash and 13.4% moisture. The experimental diets were made by adding 2.2 parts of corn oil or 2.6 parts of butter to 10 parts of the basal diet. Approximately 40% of the caloric value of these diets was thereby derived from fat. The diets were made up weekly and

TABLE I. Serum Cholesterol Levels of Swine on Control and Experimental Diets.

	Total cholesterol, mg/100 ml	t	p
Control	52 ± 21	1.47	.1–.2
Corn oil diet	65 ± 15		
Control	61 ± 23	2.38	.025
Butter diet	84 ± 18		

stored in a refrigerator. During the control period the animals were free to graze. During the experimental period the animals were housed in pairs in pens that had both inside and outside runs. They were fed once daily. At the start of the experimental period the animals weighed about 475 lb each. An effort was made to keep the body weight as constant as possible. The animals were bled from the jugular vein twice while on the basal diet. The interval between bleedings was one week. They were bled again after 3, 6, and 9 weeks on the experimental diets. They were killed and necropsied after 9 weeks on the diets. At this time 4 additional boars of the same strain and age, maintained on a conventional diet, were killed as anatomic controls. The serum was analyzed for total and free cholesterol(3), for lipid phosphorus (4), and for total lipid(5). The triglyceride was calculated by difference(5). The 2 serum samples collected during the control period and the first and third samples during the experimental period were subjected to lipoprotein fractionation by repeated ultracentrifugation at increasing solvent densities(6). The fractions were then analyzed chemically for lipid and protein content. At necropsy the heart and aorta only were examined. Tissues were fixed in formalin, sectioned with the freezing microtome, and stained with oil red O and hematoxylin.

Results. One corn-oil fed animal lost 27 lb during the experimental period. All others gained weight. The average gain for corn-oil fed animals was 31 lb and for butter fed animals was 29 lb. This gain represented approximately 5% of their original weight.

The mean serum cholesterol levels together with standard deviations for control and dietary periods are shown in Table I. Animals fed corn oil showed a slight increase over their control levels, but the difference is not significant. Animals fed butter showed a significant increase with the probability that it was due to chance 1 in 40. When the levels in the butter fed group are compared with the levels in the corn-oil fed group the p value is 0.02. The maximum rise in serum cholesterol levels occurred within the first 3 weeks of butter feeding. Thereafter the levels remained relatively constant. The cholesterol-phospholipid ratio showed no significant changes, averaging 0.84 under all conditions. The serum triglyceride levels averaged 40 mg per 100 ml. There were no significant dietary differences.

Fractionation of serum lipoproteins during the control period showed that the very low-density classes (Sf>10) carried practically all the serum triglyceride and therefore varied in concentration with the latter. As is true of all animal species so far studied, with the single exception of man, concentration of low-density lipoproteins (Sf 0-10) was less than concentration of the high-density lipoproteins. During the control period the former averaged about 84 mg per 100 ml, the latter 207. The cholesterol:phospholipid ratio in the low-density fraction (Sf 0-10) exceeded unity. In this respect swine resemble man rather than other mammals studied.

Animals on the corn oil diet showed no significant changes in lipoprotein patterns over the control period. Animals on the butter diet showed an increase in the high-density classes only. The mean increase in cholesterol content of this class was 30%, in phospholipid content 29%, and in protein content 19%.

Examination of the hearts of the 12 animals revealed no abnormalities of valves or coronary arteries. The aortas of 6 animals, however, revealed lesions. These were sharply delineated thickenings of the intima. They were longitudinal, paralleling the long axis of the aorta and varied in size up to 5 x 2 mm of the same pale color as the surrounding normal intima. They were confined to the descending thoracic aorta and appeared identical with those illustrated by Gottlieb and Lalich(1).

Microscopically these intimal thickenings showed that 2 types of cell predominated. One type resembled the histiocyte and contained in its cytoplasm varying quantities of sudanophilic material. Some were engorged with lipid. The other type of cell was round and its cytoplasm swollen with unidentified, homogeneous, unstaining material. In some lesions one type of cell predominated; in other lesions, the other type. These lesions were found in 1 of the corn-oil fed group, in 3 of the butter fed group, and in 2 of the control group.

Discussion. The feeding of butter to swine resulted in an elevation of serum cholesterol. Feeding of isocaloric quantities of fat as corn oil did not produce significant elevation in serum cholesterol levels, so the rise in the butter-fed group was presumably not caused by increased ingestion of fat *per se*. The fact that elevation in serum cholesterol was confined to an elevation in high-density lipoproteins was surprising. Manipulations of diet in man and most other experimental animals, if they affect the serum lipids at all, usually alter concentration of the low-density lipoproteins. It has been shown in the rat, however, that fasting is accompanied by an increase in high-density lipoproteins and that carbohydrate feeding is accompanied by a decrease in these same classes(7).

Summary. 1) Addition of butter to the diet of swine caused a significant elevation in serum cholesterol levels. This did not occur in other animals fed isocaloric quantities of corn oil. The increase in cholesterol was limited to the high-density (alpha) lipoproteins. 2) Necropsy after 9 weeks of experimental diets revealed lesions somewhat resembling human atheromata in 50% of aortae. The same incidence was found in control animals on a conventional diet.

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Distribution of Coxsackie Virus in Experimentally Infected Cockroaches, *Periplaneta americana*.* (23195)

ROBERT G. FISCHER AND JEROME T. SYVERTON
Departments of Bacteriology and Immunology, University of North Dakota, Grand Forks, and University of Minnesota, Minneapolis.

The transmission of the polio-encephalomyelitis group of viruses is not clearly understood(1,2). The most probable natural mode of transmission is fecal-oral dissemination with the alimentary tract as portal of entry. This hypothesis is supported by the finding of virus in feces and throat washings from patients and in sewage and flies(3). Persistence and possible multiplication of virus in non-biting flies(4) suggests that polio-encephalomyelitis viruses may escape the intestinal-oral cycle in the natural mammalian carrier by transmission through arthropods to new hosts. Incrimination of flies as potential vectors(3,4) suggests a similar role for cockroaches, since in many areas of the world the association of cockroaches with man's food and excreta exceeds that of flies. Supporting evidence is limited to recent experimental findings. Hurlbut(5) reported that after injection of human poliomyelitis virus (Lansing strain) into the haemocoele of the cockroach,

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* Aided by grant from the National Foundation for Infantile Paralysis.

Periplaneta americana, virus could be recovered 15 days later by passage of triturated whole cockroach to normal mice. He was not able to demonstrate virus in roach feces or eggs. Woke and Rosenberger(6) reported that injection of mouse strain FA encephalomyelitis virus into haemocoeles of several species of invertebrates resulted upon titration of the entire triturated invertebrate in evidence for virus survival to 28 days. The authors(7) showed that *Periplaneta americana* under experimental conditions could acquire GDVII strain mouse encephalomyelitis virus. After being fed, the virus was harbored intracorporeally for as many as 7 days and excreted daily in amounts sufficient to kill mice. Further experiments(8) with *Periplaneta americana* fed a single meal containing Coxsackie virus (Group A, Type 4, Minnesota 1 stain) proved that test roaches excreted daily for as many as 15 days sufficient virus to paralyze and kill test mice. Control experiments showed that paralysis and death of test mice resulted from infection by Coxsackie virus and not from extraneous bacterial or viral infection.

The present paper reports for the American cockroach, *Periplaneta americana,* survival of active Coxsackie virus from time of oral ingestion.

Materials and methods. Feeding of virus to adult cockroaches (*Periplaneta americana*) strapped by cellulose tape to corks(9) was accomplished with tuberculin syringe and blunt needle: a single meal of 0.2 ml of a 10% hind limb suspension from mice moribund from Coxsackie infection was fed. The diet thereafter consisted of fox chow and water. *Harvesting of viscera* was done at 4 intervals of 5 days: 3-8 cockroaches were dissected to obtain pools of viscera representing (a) gastro-intestinal tracts, (b) salivary glands, (c) reproductive organs, and (d) fat bodies and haemocoeles. Care was taken to prevent intermixing of visceral fluids and dehydration of viscera. Tissues were stored at –20°C until tested for virus. *Virus assays* were done on each 5-day pool of viscera, triturated in a mortar with 0.85% NaCl solution and antibiotics to yield a 10% tissue suspension and a final concentration of 500 units of penicillin and 100 μg of streptomycin. After 30 minutes at room temperature, suspensions were centrifuged at 700 rpm for 10 minutes. The supernatant fluid in 0.05 ml doses was injected intraperitoneally into litters of 24 to 48-hour-old Swiss albino mice.

Results. Results of 13 experiments are summarized in Tables I and II. Table II presents the findings in graphic form and includes results of periodic tests of 24-hour fecal specimens from test cockroaches.

Pooled gastrointestinal tract samples removed from cockroaches at 5-day intervals up to 20 days following a single feeding of Coxsackie virus yielded on intraperitoneal injection sufficient virus to paralyze and kill test mice in from 2 to 4 days (Table I). Passage of 1% hind-limb suspensions from dead recipient mice to new litters demonstrated lethal content of virus. *Salivary glands* were found in about half of the experiments to contain virus; in most cases, virus recovery was limited to 5-day samples. In one experiment virus was demonstrated in the 5, 10 and 20-day samples, but not in the 15-day material. *Fat bodies and haemocoeles* yielded virus 3 times in 32 attempts. The irregularity suggests cross contamination during dissection. *Reproductive organs* similarly yielded virus only a few times. *Fecal specimens* periodically collected at 24-hour intervals and inoculated intraperitoneally contained irregularly sufficient virus to paralyze and kill test mice (Table II). *Effect of feeding Coxsackie virus-fed cockroaches to adult Swiss albino mice* was determined with adult cockroaches fed a known content of Coxsackie virus as previously described. After 2 days, 4 cockroaches were fed to 2 mice. Fecal specimens representative of each 24-hour period were collected daily from the mice and stored at –20°C until tested for virus. Supernates of fecal specimens representative of 4 successive days were prepared by the method used for roach tissues: 0.05 ml doses were inoculated intraperitoneally into 24 to 48-hour-old Swiss albino mice. Sufficient virus was present in the 1st and 2nd day fecal specimens to paralyze and kill test mice; 3 and 4-day fecal specimens were negative.

Discussion. Under the described experi-

TABLE I. Recovery of Coxsackie Virus in Mice Injected with Visceral Suspensions from Virus-Fed Cockroaches.

Days after single meal	Mouse passage	Cockroach series*												
		1	2	3	4	5	6	7	8	9	10	11	12	13
(G. I. tracts)														
5	1	7/7			10/10	8/8	8/8	10/10	6/6	8/8	9/11	2/9	7/7	8/8
	2†	6/6			6/6	9/9	6/6	6/6	7/7	6/6	9/10		4/4	9/9
10	1	8/8			8/8	6/6	8/8	2/5	2/7	6/6	1/5	0/10	0/10	3/8
	2	7/7			9/9	6/6	10/10	6/6	6/6	7/7	2/6			7/7
15	1					11/11	0/7	0/12	0/7	0/7	1/5	0/7	0/7	3/8
	2					8/8					8/8			5/5
20	1					0/7	0/7					0/10	0/10	4/9
	2													7/7
(Salivary glands)														
5	1	0/5	8/9	2/5	9/9	0/5	1/9	1/9	1/8	0/6	0/7	0/8	0/7	1/5
	2		1/5		8/8		11/11	8/8	8/8					8/8
10	1		5/7	0/11	1/8	0/7	0/7	0/5	0/7	0/7	0/6	0/4	0/2	2/7
	2		9/9		0/11									7/7
15	1					0/10	0/8	0/5	0/6	0/7	0/8	0/5	1/9	0/7
	2												5/5	
20	1					0/8	0/10						0/10	2/7
	2													7/7
(Fat bodies, haemocoeles)														
5	1				5/7	1/7	8/8	0/5	0/7	0/7	0/8	0/6		
	2				9/9	0/5	9/9							
10	1				0/6	1/7	0/8	0/7	0/6	0/8	0/5	0/7	0/6	0/4
	2					0/3								
15	1					0/8	0/7	0/5	0/7	0/5	0/7	4/5	0/8	0/9
	2											9/9		
20	1					0/7	0/8						0/8	0/3
	2													
(Reproductive organs)														
5	1			6/10				2/11	0/10	0/8	0/8	1/5		0/4
	2			10/10				7/7				6/6		
10	1			0/9				0/6	0/5	0/8	0/7	0/8	0/7	3/3
	2													5/5
15	1							0/5	0/6	0/7	0/9	0/6	0/6	0/2
	2													
20	1													1/4
	2													8/8

* Mice dead of Coxsackie infection/mice inj. intrap., 0.05 ml.

† Hind limbs from mice of passage 1 employed as inoculum for passage 2.

mental conditions, samples of pooled gastrointestinal tracts removed at 5-day intervals up to 20 days from cockroaches fed a single meal containing Coxsackie virus contained sufficient virus to paralyze and kill test mice. Recovery of virus from pooled salivary glands was limited to 5-day samples with one exception when virus was recovered from 5, 10 and 20 day samples. It appears possible that a) cockroaches by feeding on mammalian excreta could acquire virus, and maintain it for a period of time, and transfer it by contamination of food, or that b) cockroaches so infected might be consumed by wild mice to transmit virus through the feces of the mice. The presence of Coxsackie virus in feces and its recovery from sewage of cities in which Coxsackie infections have occurred(3) indicates that cockroaches have opportunities for acquisition of virus. Present and previous experimental findings(7,8) suggest a possible role for cockroaches in the transmission of

TABLE II. Recovery of Coxsackie Virus from Cockroach Viscera.

DAYS AFTER SINGLE MEAL	COCKROACH SERIES												
G. I. TRACTS	1	2	3	4	5	6	7	8	9	10	11	12	13
5	■			■	■	■	■	■	■	■	■	■	■
10	■			■	■	■	■	■	■	■	●	●	■
15					■	●	●	●	●	■	●	●	■
20					●	●						●	■
SALIVARY GLANDS													
5		■	■	■	●	■	■	■	●	●	●	●	■
10		■	●	●	●	●	●	●	●	●	●	●	■
15					●	●	●	●	●	●		■	●
20					●	●						●	■
FAT BODIES AND HAEMOCOELES													
5	●			■	●	■	●	●	●	●	●	●	
10				●	●	●	●	●	●	●	●	●	●
15					●	●	●	●	●	●	■		●
20					●	●						●	●
REPRODUCTIVE ORGANS													
5			■				■	●	●	●	■	●	●
10			●				●	●	●	●	●	●	■
15							●	●	●	●	●		●
20												●	■
FECAL SPECIMENS													
1				■									
2													
3													
4												●	■
5		■		■	●	■	■	■		■		●	■
6	■		●			■				■	●		
7	●					■		■		●			
8	●					■		●					■
9	●		●	●		●		●		●			
10	●		●		●	●	■	●	●				
11	●						●						
12							■						
13							■						
14							●						
15					●						●		
20					●								

■ COXSACKIE INFECTION WITH POSITIVE SECOND PASSAGE

● NEGATIVE RESULT

sporadic Coxsackie and poliomyelitis infections in man.

Summary. Gastrointestinal tracts removed at 5-day intervals for up to 20 days from cockroaches, *Periplaneta americana,* fed a single meal of Coxsackie virus contained sufficient virus to paralyze and kill test mice. Salivary glands removed after 5 days from the test cockroaches also caused paralysis and death in test mice; in one experiment, salivary glands removed 5, 10 and 20 days after a single feeding of virus caused Coxsackie infection in test mice.

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Heat-Stability of *Candida albicans* and *Saccharomyces cerevisiae* Hemagglutinating Antigens. (23196)

RALPH A. VOGEL (Introduced by Morris Tager)
Veterans Administration Hospital, Atlanta, Ga., and Department of Bacteriology,
Emory University School of Medicine, Ga.

Erythrocyte sensitizing substances (ESS) derived from *Candida albicans* and *Saccharomyces cerevisiae* have recently been described as requiring lipid for sensitization of red cells in the hemagglutination test(1). Further studies of these antigens led to an investigation of their heat-stability. Unexpectedly it was established that these antigens prepared by similar technics react differently to heat in respect to their capacity subsequently to sensitize red cells. In the following report serologic activity of ESS from *C. albicans* and *S. cerevisiae* is compared to whole yeast-cell agglutinating antigen from these organisms before and after heat-treatment.

Materials and methods. Preparation of antigens and serologic tests employed. ESS from *C. albicans* and *S. cerevisiae* derived from liquid medium, and antisera to these organisms were obtained as described recently (1). The hemagglutination test described previously was used to test activity of these antigens(2). Whole yeast-cell antigens were prepared from cells grown at optimal temperatures on Sabouraud's dextrose agar. The cells were heat-killed at 60°C for 2 hours, washed 3 times in saline and adjusted to a No. 2 McFarland nephelometer tube. The yeast-cell agglutination test employed 0.4 ml serial dilutions of inactivated rabbit sera to

TABLE I. Serologic Activity of *Candida albicans* and *Saccharomyces cerevisiae* Antigens before and after Heat Treatment.

	Treatment of antigens					
	Unheated		Heated (70°C 48 hr)		Autoclaving	
	Antiserum		Antiserum		Antiserum	
Antigen	Cand.	Sacch.	Cand.	Sacch.	Cand.	Sacch.
Candida albicans (yeast cells)	2048*	128	2048	128		
Saccharomyces cerevisiae (yeast cells)	256	2048	256	2048		
Candida ESS						
Undil.	256	32	512	64	1024	128
1:2	128	0	256	64	256	128
1:4	32	0	32	0	128	0
Saccharomyces ESS						
Undil.	2048	256	0	0	0	0
1:2	2048	128	0	0	0	0
1:4	2048	128	0	0	0	0

* Reciprocal of highest serum dilution giving agglutination.

which were added 0.4 ml antigen. The test was incubated for 2 hours at 37°C and spun at 1000 rpm for 5 minutes. The degree of clumping indicating a positive reaction was read by gently flicking the tubes to resuspend the sediment. *Heat-treatment of antigens.* Antigens submitted to heat-treatment were either placed in a thermostatically controlled water-bath at 70°C or autoclaved at 121°C at 15 lb pressure for 15 minutes. The heated antigens were then titrated in homologous and heterologous antisera to determine any alterations in sensitizing activity.

Results. The effects of heating on yeast-cell agglutinating antigens and ESS substances are presented in Table I. Heat did not diminish serologic activity of the yeast-cell agglutinating antigens or ESS from Candida but did have a profound effect on Saccharomyces ESS. The ESS from Saccharomyces antigen was totally inactivated by 70°C for 48 hours or autoclaving while the Candida ESS showed some increase in antigen activity as a result of heating. In experiments conducted to determine rate of inactivation of the Saccharomyces antigen it was found that at 38 hours red cells sensitized with the heated material still agglutinated in homologous antiserum. This residual antibody was blocked by adding small increments of the Candida ESS antigen to the Saccharomyces antiserum, suggesting the common antigen shared by these organisms is more resistant to heat than the type-specific one. *Studies with inactivated Saccharomyces ESS.* As noted previously ESS preparations inactivated by various procedures did not suffer a concomitant loss in precipitin activity, presumably due to stability of the haptenic polysaccharide portion of the antigen(1). In the present heat experiments there was likewise no loss in either qualitative or quantitative precipitin activities of the heated Saccharomyces ESS despite total loss of its capacity to sensitize erythrocytes.

When Saccharomyces and/or Candida antiserum was treated with aliquots of the active or heat-inactivated Saccharomyces ESS to determine whether heating had altered capacity of the antigen to block the hemagglutination test, oddly enough both the heated and unheated materials precipitated large quantities of antibody from either Candida or Saccharomyces antiserum but did not remove or block all antibody which agglutinated red cells sensitized with active Saccharomyces ESS. This residual antibody was blocked by subsequent addition of Candida ESS, which indicates that it was common antibody. Why the Saccharomyces preparation did not remove all common antibody is obscure at this time, however, it is possible that common antigen was almost totally lacking in these ESS preparations.

Discussion. The probability is strong in view of recent findings that ESS preparations from Candida and Saccharomyces organisms as well as from Staphylococcus(3) contain a haptenic polysaccharide and a lipid-like material which is instrumental in effecting attachment of the polysaccharide to red cells. There appears to be further evidence in this direction as a result of heating experiments which show that Saccharomyces ESS antigen can be inactivated without altering its serologic activity as measured in the precipitin reaction. The failure of heat to inactivate the yeast-cell agglutinating antigens can be interpreted to mean that the haptenic polysaccharide, identical to the ESS polysaccharide fraction, is firmly bound to the cell surface and does not need a lipid factor to support serologic activity; it also indicates the heat stability of the haptenic polysaccharide antigen on the surface of the yeast-cell. On the other hand the Saccharomyces ESS, a cell free antigen, requires participation of lipid to develop the antigenic complex involving erythrocytes and haptenic polysaccharide and can be inactivated by heat through the lability of the lipid in this instance.

Oeding(3) has reported on the heat-lability of the Staphylococcal ESS. While it was very resistant to heat, 1 N NaOH or storage led to its inactivation without loss of precipitinogen activity. On the basis of absorption with normal red cells which led to the removal of the sensitizing factor but very little precipitinogen, Oeding suggests the presence of 2 antigens with identical specificity but only one with the ability to sensitize red cells. We have attempted to identify 2 such components in our preparations with similar technics but have found that absorption of extracts with normal red cells, which inactivates their sensitizing properties does not do so by removal of the ESS but by the blocking action of surface protein of normal red cells. The latter can be removed by deproteinization resulting in reactivation of absorbed, inactivated extracts.

It has been stated that heat is not necessary for activation of these ESS preparations (1). In the present study it was found that the Candida extracts, while not requiring heat for activation, did acquire enhanced activity as a result of heat treatment (Table I). Both Neter(4) and Landy(5) have previously noted the activating effect of heat on enterobacterial hemagglutinating substances.

C. albicans and *S. cerevisiae* share a common antigen which can be demonstrated by the gel diffusion technic(6). Likewise red cells sensitized with ESS from either organism cross react in the other antiserum which is further evidence of common antigen. While the Saccharomyces ESS activity is totally inactivated by heat for either serum, the Candida ESS is somewhat enhanced by heat in both sera. It is very likely, from these observations, that the common antigen shared by these 2 organisms is combined with different lipid carriers, one heat-labile, the other heat-stable.

These studies were initially undertaken to study the antigens of a common yeast-like pathogen such as *Candida albicans* and a saprophytic yeast *Saccharomyces cerevisiae.* While there are many striking differences already recorded regarding animal pathogenicity and morphologic features of these organisms, it is interesting to note that antigen derived by similar means from these organisms exhibit striking differences in their stability to heat.

Summary. The heat-lability of erythrocyte sensitizing substances from *Candida albicans* and *Saccharomyces cerevisiae* was examined. Heat-treatment enhanced activity of Candida (ESS) antigens while it totally inactivated Saccharomyces (ESS) antigen. The common (ESS) antigen shared by these organisms is believed to be more resistant to heat than the type-specific antigen. Heat treatment did not affect activity of the whole yeast-cell antigens of *Candida albicans* or *Saccharomyces cerevisiae.*

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Antigenicity of Hyaluronic Acid.* (23197)

Robert W. Quinn and Kamala P. Singh†

Department of Preventive Medicine and Public Health, Vanderbilt University School of Medicine, Nashville, Tenn.

Studies of antigenicity of hyaluronic acid indicate that it is not antigenic by itself(1) and when coupled chemically with protein it does not act as a hapten(2). Recent work of Glynn and Holborow(3) reports the production of complete antigens from vegetable polysaccharides (chondroitin sulfate) to autoantigens by group A hemolytic streptococci (4). Although Glynn and Holborow have since indicated that they have been unable to repeat their work and that observed antibodies were directed against blood group like substances and not against chondroitin sulfate itself(5), the question of whether hyaluronic acid, and other tissue polysaccharides might become autoantigenic is important in possible interpretations of the pathogenesis of rheumatic fever. It is the purpose of this paper to report attempts to render hyaluronic acid antigenic.

Materials. Hyaluronic acid was prepared from human umbilical cords according to method described by Jeanloz and Forchielli (6), and digested with pepsin but not with trypsin.‡ Trypsin digestion was eliminated to avoid the necessity of lowering the pH with possible denaturation of hyaluronic acid. Remaining proteins were eliminated by Sevag's procedure(7) which was performed at least 10 times. Sulfur-containing compounds were removed by treating the hyaluronic acid solution twice with ammonium sulfate and pyridine(6). Streptococcal hyaluronic acid was prepared from the N.Y. 5 strain of group A hemolytic streptococcus, employing the method of Pierce and White(8). The small amount of residual protein was removed by Sevag's procedure. A crude extract of umbilical cords was prepared according to the method of McClean *et al.*(9). Bacteria used as antigens were 3 strains of group A beta hemolytic streptococci belonging to serological types 1, 4 and 14, and a strain of hemolytic *Staphylococcus aureus*. In preparation of materials for inoculation, the bacteria were incubated for 18 hours at 37°C in 100 ml of brain heart infusion broth, centrifuged and taken up in 25 ml of 0.85% saline. Half of this bacterial suspension was employed as antigen without exposure to hyaluronic acid, and half was centrifuged and resuspended in 10 ml of a solution of cord hyaluronic acid, 1 mg hyaluronic acid/ml of 0.85% saline. Bacteria were not exposed to streptococcal hyaluronic acid. Half of the bacteria were incubated at 37°C for 1 hour with hyaluronic acid *before* being heat-killed and half *after* being heat-killed. Then these suspensions were washed 4 times in 0.85% saline. A portion of the suspension in contact with hyaluronic acid before killing was not washed to test any possible effect of washing on antigenicity. Careful controls were observed to insure pure cultures. The cord hyaluronic acid and streptococcal hyaluronic acid injected without bacteria contained 1 mg and 1.3 mg hyaluronic acid respectively/ml of

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* Aided by grant from S. E. Massengill Co.

† Present address: Government Degree College, Nainital, U. P., India.

‡ Had trypsin digestion been done it is possible that the numerous attempts to remove proteins by Sevag's procedure might have been unnecessary. It seems likely that each Sevag procedure removed with it some of the hyaluronate, thus accounting for the very small yield of 255 mg from a starting material of 2000 g of human umbilical cords which had been stored in acetone.

solution. Group A, type 1 strain had been recovered from a patient with pharyngitis about 3 weeks preceding onset of rheumatic fever; this strain produced streptolysin "O", streptokinase and hyaluronic acid, but no hyaluronidase. Strain A-14 was recovered from a patient with pharyngitis about 2 weeks preceding onset of rheumatic fever and produced streptolysin "O", streptokinase, hyaluronic acid and a small amount of hyaluronidase after 7 days' incubation. The source of group A, type 4 was unknown but it produced relatively large amounts of hyaluronidase. The strain of *Staphylococcus aureus* was recovered from a patient with infected skin ulcer.

Methods. Intravenous inoculation procedures. Young adult rabbits weighing between 5½ and 6½ lb were injected intravenously in the marginal ear vein with antigens indicated in Table I. Three rabbits were used to test each antigen. Each rabbit received 1 injection of 0.5 ml of antigen daily for 3 days during the first week. They were allowed to rest 4 days, then received 1 ml daily for 3 days, followed by 4 days' rest. This routine was followed for 4 more weeks. The dose of hyaluronic acid antigens was adjusted so that each injection after the first week contained 1 mg of hyaluronic acid. After 6 weeks the rabbits were reinjected following the same schedule for 3 more weeks. The seventh course of injections was given subcutaneously in a dose of 0.5 ml; all subsequent injections consisted of 1 ml intravenously. Each rabbit received a total of 27 injections. The rabbits were bled from the ear vein 1 week after completion of the third course of injections, 2 days after completion of the sixth course and 8 days after the ninth course. The serum was separated and Merthiolate added to a strength of 1:10,000 before storing at 4°C-8°C. *Intramuscular and subcutaneous inoculation procedures.* Antigens given by these routes are listed in Table I. The hyaluronic acid or crude extract content of each antigen was 1 mg/ml. The dose was 0.5 ml 3 times weekly for an average of 23 injections. Procedure for administering these antigens differed from that described above in that half the animals received bacteria which were heat-killed before contact with hyaluronic acid or crude extract and half received bacteria which were killed after contact. In this way the possible effect of any hyaluronidase elaborated by group A, type 4 hemolytic streptococci could be observed. Each rabbit was bled before any antigen was given, at 2 week intervals during antigen administration, and 10 days following last injection. *Test for precipitating antibodies.* An optimal proportion technic was employed in which sera were tested undiluted, 1:4, 1:8 and so on to a dilution of 1:64. The antigens, 1 mg hyaluronic acid or crude extract/ml of 0.85% saline, were diluted identically. Each serum dilution was tested with each antigen dilution beginning with undiluted serum plus all antigen dilutions, etc. Dilutions of sera and antigens were made in small serological tubes. The serum was first taken up in a capillary tube to a height of about 1 cm, then overlaid by an equal volume of antigen. The tubes were incubated at 37°C for 2 hours after which they were placed in refrigerator at 4°C-8°C overnight. They were observed for evidence of precipitate during and after 37°C incubation and after 4°C-8°C incubation. *Test for antigroup-specific C carbohydrate.* Streptococcal C carbohydrate was extracted according to the procedure of Swift, Wilson and Lancefield(10). Serum from each rabbit which had received streptococci intravenously was tested for presence of antibodies against the group-specific C carbohydrate. *Test for antitype-specific M protein.* Sera from rabbits which had received streptococci by various routes were tested for precipitating antibodies against M protein. M protein was extracted from each strain of hemolytic streptococcus according to method described by Lancefield(11). *Test for skin sensitivity.* Each rabbit was skin-tested with 0.1 mg in 0.1 ml of the antigen which it received: umbilical cord hyaluronic acid, streptococcal hyaluronic acid, or crude extract. Injection sites were observed immediately and at 24, 48 and 72 hours for erythema and induration. *Test for non-precipitating, skin-sensitizing antibodies.* A test for non-precipitating, skin-sensitizing antibodies was performed with sera of rabbits which had received either

TABLE I. Antigens Injected, Route of Administration, and Antibody Response.

Materials injected	No. of animals inj.	Route of inj.	Antibody determinations						
			Pptn. ab. against hy. ac. cord	Pptn. ab. against hy. ac. strep.	Pptn. ab. against cr. ext.	Skin sensitivity	Skin-sens. non-pptn. ab.	Anti C CHO ab.	Anti M prot. ab.
Streptococcus A-14	3	I.V.	0			0	0	+	+
" A-1	3	"	0			1–e.	0	+	+
Staphylococcus aureus	3	"	0			"	0		
Hy. ac. (umbilical cord)	3	"	0			2–e.	0		
" " (streptococcal)	3	"		0		1–in. 1–e. & in.	0		
Streptococcus A-14 + hy. ac.	3	"	0			0	0	+	+
" A-1 + " "	3	"	0			1–e.	0	+	+
Staphylococcus aureus + hy. ac.	3	"	0			"	0		
Streptococcus A-14 + hy. ac. unwashed	3	"	0			0	0	+	+
Hy. ac. (umbilical cord)	1	I.M.	0			0	0		
" " + A-4, killed before contact	1	I.M.	0					+	+
	1	S.C.	0					+	+
" " + " contact before killed	1	I.M.	0			0		+	+
	1	S.C.				0		+	0
" " + staph., killed before contact	1	I.M.	0			0			
" " + " contact before killed	1	S.C.	0			0			
Crude extract	1	I.M.			0	0			
" " + A-4, killed before contact	1	"			0	1–e. & in.		+	+
" " + " contact before killed	1	"			0	"		+	+
" " + staph., killed before contact	1				0	0			
Total No. of rabbits	39								

Hy. ac. = Hyaluronic acid; Cr. ext. = Crude extract from human umbilical cords.

Killed before contact = Bacteria were heat-killed before exposure to substrate. Contact before killed = Bacteria were exposed to substrate, then heat-killed. Numbers in column under skin sensitivity refer to No. of rabbits showing erythema, induration or both.

I.V. = Intravenous; I.M. = Intramuscular; S.C. = Subcutaneous; in. = induration; e. = erythema.

streptococcal or umbilical cord hyaluronic acid intravenously with or without bacteria, employing the Prausnitz-Küstner technic. Test sites were prepared by injecting 0.1 ml of each rabbit's serum intradermally into a normal rabbit. Forty-eight hours later, each prepared site was injected in its center with 0.01 ml of the hyaluronic acid which had been employed to vaccinate the animal supplying the serum. The results were read in 30, 45 and 60 minutes.

Results. None of the rabbits had any noticeable physical reactions following injections of antigens. Immunologic results are shown in Table I. None of the rabbits developed the slightest trace of precipitating antibodies against either umbilical cord hyaluronic acid or streptococcal hyaluronic acid. The sera of rabbits which had received crude extract showed a heavy precipitate which appeared very rapidly after overlaying with the crude extract solution. However, in testing sera from these rabbits before they had received any crude extract, the same heavy precipitate was observed. Therefore, whatever this precipitate was, it was not due to precipitating antibodies acquired during administration of crude extract.

Skin tests in rabbits inoculated intravenously, using hyaluronic acid from umbilical cords or from streptococci as the skin test material, revealed slight erythema lasting for 2 days about the injection site in 1 rabbit which had received streptococcus A-1, and in 1 which had received *Staphylococcus aureus*. A large area of erythema, fading gradually in 3 days, was observed about the injection site in 1 rabbit which had received umbilical cord hyaluronic acid, and a small area of erythema lasting 1 day was seen in another rabbit which had received the same antigen. A small raised area of induration, 2-3 mm in diameter, lasting for 2 days, and a similar "lesion" plus a small amount of erythema was observed in 2 rabbits which received streptococcal hyaluronic acid. Erythema fading in 3 days was observed in 1 rabbit which received streptococcus A-1 plus hyaluronic acid, and in 1 which received *Staphylococcus aureus* plus hyaluronic acid. Erythema 10 x 10 and 10 x 12 mm was observed at 1 hour in 2 rabbits which received crude extract plus streptococcus A-4. In 24 hours the erythema measured 10 x 11 and 7 x 7 mm and there were small areas of induration 4 x 4 and 3 x 3 mm. The induration remained for several days. The significance of all the skin reactions is not clear. Had induration appeared at all injection sites there would have been no question that these reactions were indicative of a reaction of the tuberculin type, but its absence in all but 4 animals leaves some doubt as to the significance of erythema alone as a measure of sensitivity.

The results of the tests for non-precipitating, skin-sensitizing antibodies were entirely negative during the 1 hour period of observation.

Sera from rabbits which had received streptococci incubated with hyaluronic acid were tested for precipitating antibodies against M protein of types 1, 4 and 14. After the last injection, large amounts of precipitating antibody against type-specific M protein were present in sera of all but 1 of the rabbits which had received streptococcus A-1 or A-14 alone or in combination with hyaluronic acid. A very small amount of precipitin against type 4 M protein was present in sera of 4 of 6 rabbits which received type 4 streptococci with hyaluronic acid or crude extract. Precipitating antibodies against an acid extract of type 4 containing group-specific C carbohydrate were present in moderate amounts in sera of all rabbits which received type 4. The ability of this strain of group A, type 4 to stimulate production of anti-M antibodies in rabbits must be of a low order.

Discussion. The results of these experiments indicate that by itself, hyaluronic acid derived from human umbilical cord and from group A hemolytic streptococci does not stimulate in rabbits the production of precipitating or non-precipitating, skin-sensitizing antibodies, nor does umbilical cord hyaluronic acid become a hapten as does agar(3) when exposed to group A hemolytic streptococci or *Staphylococcus aureus*. The same can be said for crude extract of umbilical cord. The results were the same for all 3 routes of administration. Why hyaluronic acid did not stimulate production of these antibodies is not explained by these experiments, but it is of interest that Glynn and Holborow§ also failed to produce antibodies to hyaluronic acid using these technics. Possibly the hyaluronic acid was depolymerized or was not bound tightly to bacterial cells and was washed off in preparing the antigens, although these same workers made the observation that live group A streptococci, having once been in contact with agar or alginate solution, acquired antigenicity for these haptens, which was retained despite repeated washing of the live cocci(4). Furthermore, in the present experiment 3 rabbits which received immunizing doses of streptococci and hyaluronic acid which were not washed, did not develop antibodies against hyaluronic acid.

The production of antitype-specific M protein and antigroup-specific C carbohydrate antibodies in rabbits inoculated with streptococci is evidence that these rabbits had received a dose of at least 2 antigens large enough to stimulate antibody production. If hyaluronic acid were antigenic, it would seem that there had been ample time for antibodies to appear. The possibility that the hyaluronic acid may have been destroyed by hyaluronidase produced by the streptococci does not seem valid because type 1 did not produce this enzyme and type 14 did so only in very small amounts after a 7 day incubation period. Rabbits which received hyaluronic acid and crude extract which had been incubated with hyaluronidase-producing type 4 streptococci both before and after being heat-killed did not produce antibodies either, so apparently hyaluronidase did not interfere with any potential antigenicity of the connective tissue components.

The question of production of skin sensitivity is not so clear. The presence of small areas of induration and erythema in the skin of 2 rabbits receiving streptococcal hyaluronic acid would suggest that these rabbits might have developed some tuberculin-like sensitivity. The same might be said of the 2 rabbits which received crude extract plus type 4 streptococci and developed erythema and slight induration 24 hours after test injection.

Summary. Antigens were prepared from human umbilical cord hyaluronic acid, streptococcal hyaluronic acid, group A hemolytic streptococci and *Staphylococcus aureus* and these same bacteria incubated with human umbilical cord hyaluronic acid. Rabbits were injected intravenously, intramuscularly and subcutaneously with these antigens. Neither precipitating nor non-precipitating, skin-sensitizing antibodies against hyaluronic acid developed. Anti C carbohydrate and anti M protein antibodies were produced in the animals which received streptococci, indicating that the rabbits had received enough of at least 2 antigens, one a carbohydrate the other a protein, to stimulate antibody production. The development of skin sensitivity to hyaluronic acid was suggested but by no means proved. These experiments furnish further evidence that hyaluronic acid is probably not antigenic by itself, nor does it act as a hapten capable of adsorption to group A hemolytic streptococci or *Staphylococcus aureus*.

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§ Personal communication.

Effect of Nitrogen Mustard on Experimental Tuberculosis.* (23198)

Scott V. Covert, Kenneth B. Olson and Marilyn Maisel
(Introduced by A. W. Wright)
Departments of Microbiology and Medicine, Albany Medical College, N. Y.

Since Hektoen and Corper's report(1) it has been known that mustard gas inhibits the formation of antibodies. With the introduction as therapeutic agents of the closely related nitrogen mustards, usually methyl bis (beta chlorethyl) amine hydrochloride (HN_2), by Gilman and Philips(2), the possibility of lowering resistance to infection or producing dissemination of an infectious process already present was postulated. HN_2 has found its greatest usefulness in the treatment of malignant lymphomas and at least one of these conditions, Hodgkin's disease, is associated with active tuberculosis frequently enough for treatment with this agent to pose a serious problem. Gellhorn and Collins(3) have reported one case of Hodgkin's disease in which they suggest that tuberculosis may have been aggravated by HN_2 treatment. One of us is familiar with 2 similar cases in which treatment with HN_2 was followed by rapid dissemination of tuberculosis. In none of these cases was there adequate study of the pre-treatment status of the pulmonary tuberculosis, and the possibility of rapid spread of tuberculosis in Hodgkin's disease without treatment is a possibility. Because of the consideration that HN_2 may be a factor in decreasing resistance to tuberculous infection, the following experiments were devised:

Methods. Guinea pigs protected by BCG were inoculated with virulent tubercle bacilli and later treated with HN_2 to determine the effect of the agent on the quiescent foci. At first it was decided to use a weight dose of HN_2 comparable to that used in humans but it was found that 0.1 mg/kilo given intraperitoneally for 6 days had little or no effect on the peripheral leukocyte count of guinea pigs. Accordingly, the dosage was gradually increased to 0.3 mg/kilo for 6 days, which was adequate to produce leukopenia but did not cause death of the animals. Forty-two guinea pigs weighing approximately 300 g each were employed. All animals were initially tested with 0.1 ml of a 1:10 dilution of "Old Tuberculin" (O.T.) and found to be negative. The animals were then divided into 6 groups of 7 animals each and treated as follows: Group I:BCG and *Mycobacterium tuberculosis* strain $H37R_v$; and HN_2; II:BCG and $H37R_v$; III: $H37R_v$ and HN_2; IV:BCG and HN_2; V:$H37R_v$ alone; VI:HN_2 alone. The various agents and treatments were administered as follows: (a) Animals receiving BCG were injected in the thigh with 0.1 ml of standard suspension.† After 30 days these animals were tested with O.T. and all were found to be tuberculin positive. (b) 60 days after BCG injection animals receiving $H37R_v$ were inoculated intramuscularly in the thigh with 0.1 ml of suspension containing 3 mg wet weight of bacteria. (c) Nitrogen mustard was given intraperitoneally, 0.3 mg/kilo on 6 consecutive days. This was started 15 days after injection of $H37R_v$. (d) Groups III through VI served as controls. (e) Total leukocyte counts, hemoglobin determinations and differential counts were performed on animals in Group VI immediately before the first injection of nitrogen mustard, 2 days after the last injection, and every 7 days thereafter for 3 weeks, 5 determinations in all being made of each animal. Blood was obtained by cardiac puncture. Two animals died during the course of bleeding. (f) Thirty days after inoculation with $H37R_v$, all animals, except those of Group VI, were sacrificed and autopsies performed. The animals were inspected for evidence of tuberculosis, and any lesions which were equivocal were removed for microscopic examination. A modification of the Steenken and Baldwin(4) and Feldman(5) schemes for recording experimental

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† Obtained through the courtesy of N. Y. State Department of Health, and supplied by Dr. J. D. Aronson, Henry Phipps Institute.

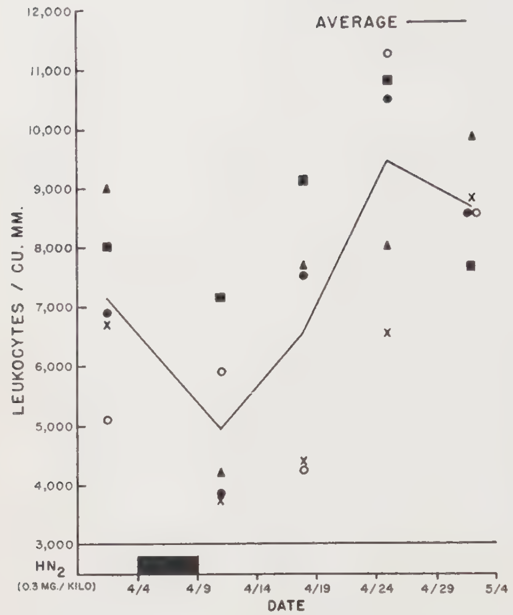

FIG. 1. Group VI. Effect upon leucocytes of 0.3 mg/kilo of nitrogen mustard given intraperitoneally on 6 successive days to guinea pigs. Each symbol represents a single animal. A higher dosage resulted in significant mortality. Lower doses were without hematological effect. Two animals died during bleeding and are not included.

tuberculosis in guinea pigs was used. Extensive involvement of an organ or regionl lymph nodes was graded as 4 with successively less involvement graded down to 0 for no evidence of lesions. The parts inspected and graded were the injection site, inguinal nodes, retroperitoneal nodes, mediastinal nodes, axillary nodes, spleen, liver, and lungs. (g) Total involvement within a group was computed and tests for statistical significance of means were performed.

Results. On a dose regimen of 0.3 mg HN_2/kilo body weight given intraperitoneally on 6 consecutive days it was possible to produce significant lowering of the total leukocyte count in groups of guinea pigs similar to those employed in the experiment (Fig. 1). The normal range of leukocyte counts in guinea pigs is between 5,000 and 10,000 cells/cu mm. While average counts are only slightly below the normal minimum, it can be seen that in 4 of the 5 individual animals there was a significant drop. Because the bleeding schedule for blood counts was set at 7-day intervals, it was conjectured that blood loss might affect the counts. Another group of animals not subjected to HN_2 treatment and bled at 7-day intervals did not reveal any decrease in total leukocytes.

The results given in Table I disclose no demonstrable difference in amount or spread of tuberculosis in groups of treated and untreated animals. The findings in various control groups substantiate the effectiveness of the various agents used.

Discussion. Various explanations for resistance of previously infected subjects to dissemination of tubercle bacilli or reinfection or reactivation of disease under certain conditions have been proposed. Evidence supporting or disproving these various explanations has been conflicting and the problem has not yet been solved. Therefore, speculation as to the reasons for the observed exacerbation of quiescent tuberculosis following treatment with HN_2 has not been attempted. What we have attempted was to ascertain if the course of experimental infections in animals would be affected by treatment with HN_2. It was postulated that when dissemination of disease in patients followed administration of HN_2, resistance had previously been good and that subsequent spread was due to a breakdown of that resistance. In Groups I and II in our study an attempt was made to set up similar conditions. When Group I animals were subjected to challenge with HN_2 no measurable difference in dissemination was observed between these animals and those of Group II. The other groups included in the study served as controls for the variables in the experiment.

In a previous experiment with larger num-

TABLE I. Mean Amount of Tuberculosis Observed in Groups I through V.

Groups	I	II	III	IV	V
Injection site	4.0	4.0	4.0	0	4.0
Inguinal nodes	.3	1.0	1.1	0	1.3
Retroperitoneal nodes	.8	.7	2.1	0	2.0
Mediastinal nodes	.7	0	1.7	0	2.0
Axillary nodes	0	0	0	0	.1
Spleen	0	.1	2.1	0	2.7
Liver	0	0	.4	0	1.3
Lungs	0	0	0	0	.8
Total	5.8	5.8	11.4	0	14.2

Group I—BCG, $H37R_v$, HN_2. Group II—BCG, $H37R_v$. Group III—$H37R_v$, HN_2. Group IV—BCG, HN_2. Group V—$H37R_v$.

bers of animals, the dose of nitrogen mustard was smaller, having been predicated on that used in humans. While the results obtained were identical with those in the present study, they were open to question because no significant leukopenia could be demonstrated with this dose.

In the present study the dose of HN_2 was calculated to cause a significant drop of leukocytes without causing death. The difference in dosage which will accomplish this effect was found to be very critical, since the animals remained well and healthy on one dose and were killed quickly when the dose was increased very slightly. It is believed, therefore, that HN_2 was given in an amount which would affect the animals since leukopenia occurred regularly in our test group.

In Groups I and II the animals were made resistant to subsequent challenge with virulent tubercle bacilli by prior inoculation with BCG. Reference to Table I indicates that virulent organisms were confined to the injection site and adjacent nodes, a situation analogous to that of patients with quiescent tuberculosis. Treatment with HN_2 appears to have had no effect on animals thus treated, since the amount and distribution of the infection were essentially the same.

In Groups III and V the animals were also inoculated with virulent organisms but did not have the protection of BCG. The guinea pigs receiving HN_2 appeared to have less dissemination of the disease than those not receiving it. A statistical analysis of the data‡ in these groups reveals the difference between the mean amount of tuberculosis present in treated and untreated animals to have no significance.

No effect was evident when animals of Group IV, which were given BCG alone, were subjected to treatment with nitrogen mustard.

Summary. Nitrogen mustard administered intraperitoneally to guinea pigs in a dose of 0.3 mg/kilo for 6 days was sufficient to produce significant leukopenia but had no appreciable effect on amount or spread of tuberculous infection nor did it reduce the protection produced by prior vaccination with BCG.

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‡ Acknowledgment is made to Sandra Heslin, Senior Bio-statistician, Office of Program and Development Evaluation, N. Y. State Department of Health, for aid in analysis of the data.

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Method for Simultaneous Estimation of Pregnane-3α, 17α, 20α-Triol and Pregnane-3α, 17α, 20α-Triol-11-One in Urine. (23199)

M. Finkelstein and R. I. Cox* (Introduced by B. Zondek)
Hormone Research Laboratory, Hebrew University-Hadassah Medical School, Jerusalem, Israel, and Department of Veterinary Physiology, University of Sydney, Australia.

Cases of adrenogenital syndrome due to adrenal hyperplasia excrete large amounts of C-20-methyl steroids such as pregnane-3α, 17α, 20α, triol(1), and pregnane-3α, 17α, 20α, triol-11-one(2), in the urine. Although milligram amounts of pregnane-3α, 17α, 20α-triol are also excreted in cases of adrenal virilizing tumor(3) and small quantities of this steroid are present in the urine of normal persons(4), the presence of pregnane-3α, 17α, 20α-triol-11-one has been established so far only in cases of adrenal hyperplasia(2). In all the above publications the presence of both compounds was established by isolation and identification. However, until recently

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no accurate analytical method has been available which would enable an easy screening of urines for presence of pregnane-3α, 17α, 20α-triol and pregnane-3α, 17α, 20α-triol-11-one. Recently, Finkelstein and Goldberg(5,6) described a test for qualitative and quantitative estimation of pregnane-3α, 17α, 20α-triol-11-one. It was shown that pregnane-3α, 17α, 20α-triol-11-one could be visualized on filter paper by moistening the paper with 70% phosphoric acid, heating it at 87°C for 10 minutes, quickly cooling to room temperature and irradiating with U.V. light with a maximal emission at 3660Å. Under these conditions pregnane-3α, 17α, 20α-triol-11-one produces a brilliant blue fluorescence which is clearly visible with 1-2 μg of the compound/sq. cm. Combined with paper chromatography, this reaction was found suitable for detection of pregnane-3α, 17α, 20α-triol-11-one in urinary extracts.

Results. With this method pregnane-3α, 17α, 20α-triol-11-one was detected in quantities above 40 μg/24 hr urine (which is the lowest limit of the test) only in urine of patients classified as female pseudohermaphrodites and macro-genitosomia praecox due to adrenal hyperplasia. On the other hand, pregnane-3α, 17α, 20α-triol-11-one was not detected in the urine of normal persons, or of patients with apparently "normal" endocrine function but receiving large amounts of ACTH and cortisone, or of patients with virilizing and non-virilizing adrenal tumors.

This method suffered, however, from the drawback that hot acid treatment was used for hydrolysis of urinary steroid conjugates, under which conditions pregnane-3α, 17α, 20α-triol is very rapidly destroyed(7). This steroid, of interest because of its occurrence in relatively high concentration in urine of patients with adrenal hyperplasia and also in cases with virilizing adrenal tumors, is thus destroyed in the test of Finkelstein and Goldberg(6).

Since pregnane-3α, 17α, 20α-triol is readily liberated from urinary conjugates with β-glucuronidase preparations(4,8), we investigated the possibility of developing a method, using enzymic hydrolysis and suitable for simultaneous determination of pregnane-3α, 17α, 20α-triol and pregnane-3α, 17α, 20α-triol-11-one. Three conditions had to be fulfilled in order to make such a single method for both compounds workable, (1), that conjugated urinary pregnane-3α, 17α, 20α-triol-11-one could be hydrolysed with β-glucuronidase under conditions employed for the hydrolysis of bound pregnane-3α, 17α, 20α-triol; (2), that both compounds could be identified under identical conditions, and (3), that pregnane-3α, 17α, 20α-triol could be easily separated from pregnane-3α, 17α, 20α-triol-11-one.

These requirements are satisfactorily met under the following conditions: (1) Enzymic hydrolysis of urine of cases of adrenal hyperplasia under conditions suitable for liberation of pregnane-3α, 17α, 20α-triol(8) has yielded amounts of pregnane-3α, 17α, 20α-triol-11-one similar to those obtained after hot acid hydrolysis. Both a molluscan preparation of β-glucuronidase(9) and Viobin Spleen glucuronidase have been used. (2) Detection of pregnane-3α, 17α, 20α-triol may be carried out under the following conditions, identical to those used for pregnane-3α, 17α, 20α-triol-11-one(6):—5/μg of pregnane-3α, 17α, 20α-triol in methanolic solution are applied on a Whatman No. 1 (for chromatography) filter paper on an area of approx. 1 cm^2. The paper is dried on air (evaporation of the solvent may be speeded up by warming with an ordinary electric lamp) then very briefly dipped in 70% phosphoric acid, blotted between 2 sheets of filter paper to remove excess reagent, and warmed for 10 minutes in an electric oven at 87°C on a preheated enamel tray. After rapid cooling a pink fluorescence is observed with pregnane-3α, 17α, 20α-triol under U.V. light of wavelength 3660 Å, while in daylight a violet color is seen. The fluorescence is clearly visible with 3-6 μg pregnane-3α, 17α, 20α-triol/sq cm. As mentioned previously, under the same conditions pregnane-3α, 17α, 20α-triol-11-one produces a brilliant blue fluorescence visible with 1-2 μg/sq cm. (3) The separation of the 2 compounds is easily achieved using paper chromatography in solvent systems described by Zaffaroni (10), or systems similar to those. For instance, chromatography of combined pregnanetriol and pregnanetriolone with benzene

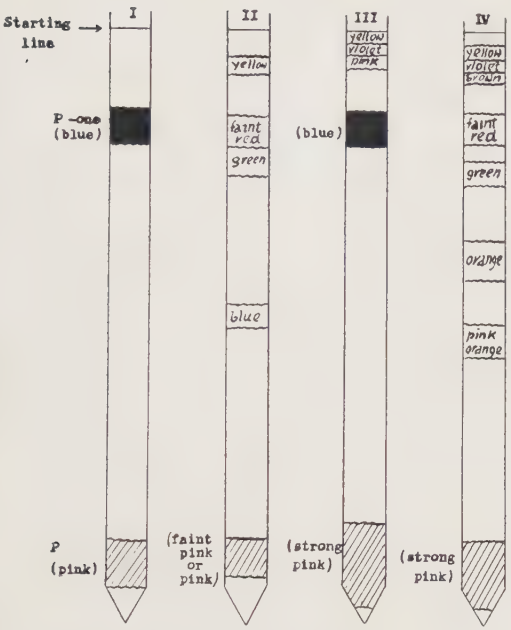

FIG. 1. Diagram of chromatograms of crystalline pregnane-3α,17α,20α-triol, pregnane-3α,17α,20α-triol-11-one and of urinary extracts. System: benzene/formamide:methanol (1:1). Running time 20 hr. Temp. 27°C. Strip I: pregnanetriol-10 μg (P) + pregnanetriolone-5 μg (P-one). Strip II: normal urine. Strip III: adrenogenital syndrome. Strip IV: adrenal adenoma.

on paper impregnated with formamide:methanol 1:1, at 27° for 20 hrs produces a separation of the compounds, as shown in Fig. 1, strip I.

Conclusions. An analytical procedure has been developed for simultaneous determination of pregnane-3α, 17α, 20α-triol and pregnane-3α, 17α, 20α-triol-11-one in urine. This procedure consists of enzymic hydrolysis, extraction of the urine with an organic solvent such as chloroform or ether, washing the extract with N/1 NaOH and water till neutral, and evaporating the extract *in vacuo* until dry. The dry residue is then dissolved in a small volume of methanol or ethanol and applied to a filter paper for chromatography for detection of both compounds through their characteristic fluorescence and estimation by comparison with standards. The significance of a method for simultaneous estimation of pregnane-3α, 17α, 20α-triol and of pregnane-3α, 17α, 20α-triol-11-one as an aid in differential diagnosis between adrenal tumor and adrenal hyperplasia is obvious, since, as pointed out above, pregnane-3α, 17α, 20α-triol is excreted in excess in cases of adrenal tumor as well as in adrenal hyperplasia, whereas pregnane-3α, 17α, 20α-triol-11-one is apparently excreted in large quantities only in cases of adrenal hyperplasia.

Typical results obtained with the method described are given in Fig. 1. Strip I shows the separation of the crystalline compounds. Strip II shows the fluorescence colors obtained with most samples of urine of normal persons. Significant is the absence of the blue spot given by pregnane-3α, 17α, 20α-triol-11-one and the presence of the faint pink or pink in position characteristic for pregnane-3α, 17α, 20α-triol, which indicates small quantities of this compound. Strip III illustrates fluorescence colors given by urinary extracts of a case of adrenogenital syndrome, due to adrenal hyperplasia (female pseudohermaphrodite). Here a distinct blue spot is visible at the same distance from the starting line as crystalline pregnane-3α, 17α, 20α-triol-11-one, and aslo a strong pink in a parallel position to crystalline pregnane-3α, 17α, 20α-triol. Strip IV is a chromatogram of a case of adrenal adenoma which shows absence of pregnane-3α, 17α, 20α-triol-11-one, but a high concentration of pregnane-3α, 17α, 20α-triol (strong pink fluorescence). A full report will be published.

Summary. A test has been described for simultaneous estimation of urinary pregnane-3α, 17α, 20α-triol and pregnane-3α, 17α, 20α-triol-11-one. After liberation from their conjugates by β-glucuronidase hydrolysis and extraction of the free compounds from urine, they are distinguished and estimated by paper chromatography and by the specific fluorescence colors which they develop on heating the paper strips, moistened with 70% phosphoric acid, for 10 minutes at 87°. Increased excretion of pregnane-3α, 17α, 20α-triol has been observed both in patients with adrenal hyperplasia and with adrenal tumors. Pregnane-3α, 17α, 20α-triol-11-one has been detected so far only in urine of patients with adrenal hyperplasia. Based on this differential excretion distinction between adrenal tumors and adrenal hyperplasia seems to be possible.

The authors wish to express their sincere thanks to Professor C. W. Emmens of Sydney for so kindly having made available full facilities for performing this work in his Department.

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Progesterone a Precursor of Testicular Androgens in Sheep.* (23200)

JAMES I. RAESIDE (Introduced by C. W. Turner)
Massey Agricultural College, University of New Zealand, Palmerston North.

Recent studies have provided evidence for a possible relation between fecal androgen and progesterone metabolism in the bovine (1). Furthermore, several androgenic compounds have been identified in the feces of a pregnant cow following the administration of progesterone(2). While feces from normal bulls and steers are inactive(3,4) it is interesting to note that progesterone administration to a bull produced statistically significant increases in the androgen content of the feces (1). *In vitro* formation of testosterone and Δ^4-androstene-3, 17-dione from progesterone has been demonstrated for rat testis(5) and human testicular tissue(6).

In view of this finding, the results of an experiment in which progesterone was given to a ram and a castrate male sheep are presented as indicating possible conversion of progesterone to androgens by the ovine testis.

Materials and methods. Collections of feces were made daily from 2 male sheep, one of which had been castrated as a lamb. The ram was a 2-year-old New Zealand Romney x Lincoln and weighed approximately 140 lb during the period of experimentation. The second animal was a New Zealand Romney wether, 14 months of age, weighing 80 lb. After preliminary collection period of one week each animal received daily during the next week a subcutaneous injection of 0.5 g and 0.25 g respectively, of progesterone in arachis oil. Total feces were dried daily for 48 hours in an oven at 45°C and ground to a fine powder in a Wiley mill. White Leghorn cockerels from the College Farm were fed the dried feces as 10% of the control ration for 21 days, starting when chicks were 4 days old. The increase in comb weight was used to assess androgen content of the fecal samples when compared with the comb response to methyl-testosterone incorporated in the feed. In a preliminary trial a sample of dried feces from a ewe in the last month of pregnancy was tested against 3 levels of methyltestosterone and one level of Δ^4-androstene-3,17-dione in the chick feed. The latter fecal sample had been stored in a dry condition for 2 years.

Results. The chick comb response revealed some androgenic activity in the feces of a pregnant ewe (Table I), whereas the response to the feeding of feces from both the untreated ram and wether was slight (Table II). When progesterone was administered, androgen excretion in the feces of the ram was very markedly increased. This finding was in con-

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TABLE I. Effect of Oral Administration of Androgens to 6 Groups of Male Chicks.

No. of chicks	Treatment	Body wt (g)	Comb wt (mg)	Comb ratio ± S.E.	Testes wt ± S.E. (mg)
15	Control	110.9	52.5	47.3 ± 5.0	21.4 ± 1.8
20	Dried feces* (pregnant ewe)	124.5	202.7	159.2 ± 17.2	27.9 ± 2.3
20	Methyltestosterone, 10 mg†	119.5	391.3	320.8 ± 28.4	24.4 ± 1.6
20	" , 20 " †	125.5	546.4	418.8 ± 32.7	19.5 ± 2.3
20	" , 40 " †	117.9	792.6	661.5 ± 38.1	20.9 ± 1.5
11	Δ^4-Androstenedione, 10 mg†	115.2	892.3	788.1 ± 71.4	14.9 ± 1.5

* Incorporated as 10% of control ration. † Wt of compound/kg feed.

TABLE II. Effect of Progesterone on Excretion of Fecal Androgens in 6 Groups of Male Sheep.

No. of chicks	Treatment	Body wt (g)	Comb wt (mg)	Comb ratio ± S.E.	Testes wt ± S.E. (mg)
20	Control	124.2	122.6	92.2 ± 12.1	27.3 ± 1.8
20	Ram dried feces*[1]	146.0	226.9	149.6 ± 15.7	36.2 ± 2.6
22	*Idem* *[2]	135.9	959.0	695.7 ± 50.5	19.8 ± .9
20	Methyltestosterone, 10 mg†	141.1	496.9	345.6 ± 21.7	31.8 ± 2.4
20	Castrate male dried feces*[1]	154.6	207.3	127.9 ± 15.6	32.2 ± 2.1
13	*Idem* *[2]	163.2	204.4	113.8 ± 15.9	31.4 ± 3.4

* Incorporated as 10% of control ration. † Wt of compound/kg feed.

[1] Collected before progesterone injections. [2] Collected during progesterone injections.

trast to the results from injecting progesterone in the castrate male sheep (Table II).

When comb growth was greatest there was a depression of testes weight(4), but hypertrophy of the combs was not always associated with a decrease in testes weight. The activity of Δ^4-androstene-3,17-dione was much greater than methyltestosterone in causing an increase in comb weight.

Discussion. A difference in the ability to transform progesterone to fecal androgen seems apparent in the present data from an experiment with an entire and castrate male sheep. The amount of progesterone administered daily to the ram was twice that given to the castrate animal but so, also, was the approximate body weight. The data show a greater power of conversion of progesterone to androgen in the male with testes.

Since oral administration of progesterone to chicks does not stimulate comb growth (1,4), no significant activity would result from excretion of progesterone as such by the sheep. Conversion of progesterone to androgens by microorganisms of the gastrointestinal tract(1,2) might explain the slight androgenic activity in the feces of castrate sheep, but would hardly account for the great increase in fecal androgen of the ram. It would appear that the tissues of the ram played a major part in transformation of progesterone to androgenic compounds in the feces of this animal. While adrenal tissue may be implicated(7), it seems likely that activity of the testes was of more significance. Further support is given to this conclusion by the recent demonstration of conversion of progesterone to androgenic compounds *in vitro* by rat and human testis(5,6). The present data therefore provide evidence that progesterone may be a precursor of testicular androgens *in vivo.*

Summary. Daily administration of progesterone by subcutaneous injection to a ram and a castrate male sheep for one week resulted in a great increase in the fecal androgenic activity of the ram. This observation suggests that progesterone may serve as a precursor of testicular androgens in the sheep. Some androgen excretion in the feces of a pregnant female sheep was noted but little fecal androgen from untreated males.

The author wishes to thank Professor A. L. Rae and Mr. T. S. Ch'ang for statistical analysis.

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Intracellular Distribution of Proteolytic Enzymes in Rat Liver Tissue.* (23201)

JOHN T. FINKENSTAEDT (Introduced by C. N. H. Long)
Department of Biochemistry, Yale University, New Haven, Conn.

The presence of intracellular proteolytic activity in mitochondria isolated from rat liver homogenates has been demonstrated(1, 2). deDuve *et al.*(2) utilized a modified hemoglobin substrate that may have been attacked by many enzymes. Maver and Greco (1) used benzoyl-L-argininamide, but their mitochondrial preparations were contaminated with large cytoplasmic granules, probably nuclear material.

Two intracellular proteinases have been extensively purified and studied(3,4,5). These enzymes have been designated cathepsins B and C. Previous work has shown that a synthetic substrate suitable for the assay of cathepsin B is benzoyl-L-argininamide, while one for cathepsin C is glycyl-L-argininamide, while one for cathepsin C is glycyl-L-tyrosinamide. This paper offers additional information about localization of the intracellular proteolytic enzymes of rat liver obtained by using synthetic substrates and cellular fractionation procedures.

Materials and methods. Liver tissue from 170 g Wistar strain rats was used in all experiments. Four grams of liver were homogenized in 36 ml of sucrose solutions by the method of Potter and Elvehjem(6). The 0.25 M sucrose plus 7.5% polyvinylpyrrolidone (PVP) solution of Novikoff† and 0.88 M sucrose were used as fractionation media. Fractionation of the homogenates was obtained by differential centrifugation following the schemes presented by Schneider and Hogeboom(7) with minor modifications. Following fractionation, 5 components were available for testing: 1) homogenate, 2) nuclei and debris, 3) mitochondria, 4) microsomes, and 5) supernatant fluid. 0.88 M sucrose was added to bring the final volume of each fraction to 40 ml. Immediately thereafter, the enzyme activity was determined at 37°C in a reaction mixture with the following composition: 1) 0.4 ml of citrate buffer 0.04 M, 2) 0.1 ml of cysteine hydrochloride 0.04 M, 3) 0.2 ml of the above components, 4) 0.2 ml of substrate 0.05 M, and 5) sucrose to a final volume of 1 ml and a pH of 5. Samples were removed from the incubation mixtures at one and 2 hours for analysis in duplicate Conway vessels. The analysis was performed with the use of a glass electrode system and the addition of alkali by a microcapillary burette calibrated in lambdas(8). The substrates utilized were benzoyl-L-argininamide hydrochloride monohydrate (BAA), and glycyl-L-tyrosinamide acetate (GTAA). The rates of hydrolysis were linear in all instances. Results are listed as cathepsin units (C.U.). A cathepsin unit is defined as that amount of enzyme or enzyme-containing material that provides 1% hydrolysis per minute. Analysis for protein nitrogen was done by the micro Kjeldahl technic(8) or the biuret method(9).

Results. The data given in Table I indicate that the enzymes which hydrolyse BAA

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† Reported in *Fed. Proc.,* 1956, v15, 322.

TABLE I. Cathepsin B and C Activity of Normal Rat Liver Fractions.

		Cathepsin B*				Cathepsin C†			
		A‡		B§		A‡		B§	
Fractions	N, mg/ml	C.U.	C.U./mg $\times 10^2$	C.U.	C.U./mg $\times 10^2$	C.U.	C.U./mg $\times 10^2$	C.U.	C.U./mg $\times 10^2$
Homogenate	16.0	.08	.5	.1	.6	.12	.7	.12	.7
Nuclei + debris	5.6	.04	.7	.04	.7	.03	.5	.03	.5
Mitochondria	3.4	.27	8.0	.29	8.5	.16	5.0	.17	5.0
Microsomes	2.4	.04	1.7	.03	1.3	.03	1.3	.04	1.7
Supernatant fluid	4.6	.04	.9	.03	.6	.03	.6	.03	.6
Mitochondria + microsomes		.26				.17			
Mitochondria + supernatant fluid		.04				.04			
Mitochondria + microsomes + supernatant fluid		.06				.05			

* Substrate, .05 M BAA. † Substrate, .05 M GTAA. ‡ .88 M sucrose fractionation.
§ .25 M–PVP sucrose fractionation.

and GTAA are localized in the mitochondrial fraction of liver homogenates. This hydrolytic activity is greater than the homogenate and/or any of the other fractions. This phenomenon may be the consequence of the removal of an inhibitor or inhibitors for the hydrolysis of GTAA and BAA present in the supernatant fluid of the same homogenates. Table I demonstrates this inhibition by showing the marked C.U. reduction produced by adding supernatant fluid to reaction mixtures containing mitochondria.

The inhibiting substance for the hydrolysis of BAA is stable to heat (90° for 10 minutes) and to freezing. Its activity is recovered in the dialysing fluid following dialysis against an equal volume of 1% sodium chloride for 24 hours at 0°C. This inhibiting activity is lost if the supernatant fluid is either treated with an equal volume of 12 N HCl at 110°C for 24 hours or reduced to an ash.

Additional studies indicate that the inhibitor present in the supernatant fluid is as effective in preventing the hydrolysis of BAA by partially purified cathepsin B as it is in preventing the hydrolysis of BAA by the mitochondrial fraction. It is far less effective in preventing the hydrolysis of BAA by crystalline trypsin. Crystalline soy bean trypsin inhibitor does not alter the hydrolysis of BAA by the mitochondrial fraction of rat liver homogenates.

The fractionation media utilized were selected because of their demonstrated ability to keep mitochondria as close to the intact state as possible. However, incubation media and conditions probably lead to disintegration of the mitochondria. Therefore, it is possible that hydrolytic activity for BAA and GTAA is associated with the mitochondrial fraction. It is not yet possible to postulate the exact position of this activity in or on the mitochondria.

Discussion. The results presented support the finding of deDuve showing that intracellular proteolytic activity is associated with the mitochondrial fraction of rat liver homogenates. Furthermore, the substrates utilized in these experiments are more specific for the cathepsins B and C of Fruton and others (3,4).

Studies on partially purified cathepsin B by Greenbaum and Fruton(3) have demonstrated that crystalline soy bean trypsin inhibitor had no significant effect on rate of action of cysteine activated cathepsin B on BAA. These investigators observed a nonlinearity between cathepsin B concentration and its activity. This was attributed to the presence, in spleen extracts, of some inhibitor.

The results here presented show that the mitochondrial substance acting on BAA has properties similar to cathepsin B as follows: 1) it hydrolyses BAA under the same conditions, 2) it is not inhibited by crystalline soy bean trypsin inhibitor, 3) it is inhibited by the supernatant fluid substance. The demonstration of this inhibitor for hydrolysis of

BAA may be an explanation for the findings of Greenbaum and Fruton.

Hydrolysis of GTAA by the mitochondrial fraction of liver homogenates suggests that cathepsin C is associated with this same particulate. Cathepsin C has an optimal hydrolytic activity at pH 5.2. From pH 6.6 to 7.8 extensive transamidation has been observed with progressively less hydrolysis(10). Using the preparations described in this article one will be able to test cytoplasmic particles for transamidation and other transfer reactions ascribed to the cathepsins. Their role in the nitrogen metabolism of normal cells may well be larger than the peptidase activity demonstrated by the results presented.

Summary. The mitochondria of rat liver homogenates contain proteinases similar to cathepsins B and C. An inhibitor for the hydrolytic activity of cathepsin B is present in the supernatant fluid of the same homogenates.

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In vitro Response of Functional Experimental Adrenal Tumors to Corticotropin (ACTH).* (23202)

ARTHUR I. COHEN, ERIC BLOCH[†] AND EVEMARIE CELOZZI
(Introduced by Jacob Furth)
Children's Cancer Research Foundation, Children's Medical Center, Boston, and the Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

Two secretory adrenocortical tumors are being reported; one originated in an irradiated mouse and the other in a radiothyroidectomized rat(1). The animals bearing the tumor transplants secreted steroids with glucocorticoid and mineralocorticoid but no gonadal activities. The urine of tumor-bearing mice contained an increased amount of 17-ketosteroids, particularly the 11-oxygenated 17-ketosteroids (Bloch and Cohen, unpublished data). These tumors grew in hypophysectomized animals. The adrenals of unoperated hosts were markedly atrophic indicating inhibition of ACTH discharge. In the following experiments the *in vitro* responsiveness of mouse and rat adrenal tumors to ACTH was studied in comparison with normal adrenal tissues and the nature of the steroidal secretions was investigated.

Materials and methods. Adrenal tissues. The previously described mouse strain 2 and rat strain 1 adrenal tumors were used(1). Normal adrenals were obtained from 3-month-old animals of the same strains (LAF$_1$ mice and WR rats). All animals were killed by decapitation. Tumors measuring 2-4 cm in diameter were excised and placed in cold Krebs-Ringer bicarbonate buffer, pH 7.4, fortified with 200 mg % glucose(2). Tumor slices were cut free hand with a razor and placed in a Petri dish containing cold buffer.

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* Work supported by grant of The N.I.H.

[†] Present address: Children's Cancer Research Fn., Boston, Mass.

TABLE I. *In Vitro* Response of Male LAF_1 Mouse and WR Rat Adrenal Tumor Slices to ACTH.

Tumor size[*] (cm)	Treatment	mU ACTH per 100 mg	µg equiv. cortisol/100 mg/2 hr No ACTH	µg equiv. cortisol/100 mg/2 hr ACTH added	Stimulation ratio[†]
Mouse					
4	None	200	1.8	12.6	7.0
2.5	”	100	3.3	15.2	4.7
3.5	”	50	2.6	15.4	5.9
2.5	”	60	2.4	15.2	6.4
3	”	45	1.7	12.0	7.1
3	”	25	1.4	11.0	8.1
3	”	25	5.3	13.9	2.6
		Avg	2.6 ± 1.3[‡]	13.6 ± 1.8	6.0
Rat					
2	None	150	3.4	11.3	3.3
3	”	130	3.3	17.8	5.4
5	”	130	.9	17.9	19.9
2.5	”	130	1.7	17.9	10.5
3	”	130	1.8	23.6	13.1
2.5	Adrenalectomy	140	.6	16.5	27.6
2	Gonadectomy	140	1.5	17.0	11.3
		Avg	1.9 ± 1.1	17.4 ± 3.6	13.0

[*] Estimated avg tumor diameter.

[†] Stimulation ratio = $\frac{\mu\text{g equiv. cortisol secreted with ACTH}}{\mu\text{g equiv. cortisol secreted without ACTH}}$.

[‡] Mean ± stand. dev.

The normal adrenals were dissected, carefully cleaned and cut in halves on buffer moistened filter paper. *Tissue incubation and steroid extraction.* The *in vitro* method of Saffran and Schally for bioassay of corticotropin was modified to measure the steroid production by tumor slices and normal adrenal segments(3). Approximately 30-50 mg of normal adrenals (about 10 mice or 1 rat were necessary) or 100 mg of tumor slices were blotted on filter paper, weighed accurately on a Roller-Smith torsion balance and transferred to a 10 ml Erlenmeyer flask containing 3 ml of buffer. The flasks were flushed with a 95% O_2-5% CO_2 gas mixture, stoppered and shaken in a water bath at 38°C for 1-1½ hours. At the end of this preincubation period, the buffer was removed and 3 ml of fresh buffer were added to rewash the slices in order to eliminate all traces of any interfering substances. The flasks received 2.9 ml of buffer and various concentrations of U.S.P. Reference Standard Corticotropin dissolved in 0.9% saline, the final volume being 3.0 ml. The samples were again flushed with the gas mixture and incubated for 2 hours in a shaking water bath at 38°C. At the end of the incubation period, 2.5 ml of buffer were transferred from each flask to a glass stoppered 15 ml centrifuge tube containing 2.5 ml of purified methylene chloride. The tubes were well shaken and centrifuged at 2500 rpm to separate the 2 phases. After removal of the lower phase, this extraction procedure was repeated with 2.5 ml of methylene chloride to insure complete removal of the steroids from the buffer. The combined extracts were evaporated to dryness and the residue redissolved in 1 ml or 2 ml of purified 95% ethanol. All determinations were done in duplicate or triplicate. *Estimation of Δ^4-3-ketosteroids.* The optical densities of aliquots of the ethanolic extracts were measured at 242 mµ in the Beckman model DU ultraviolet spectrophotometer against an ethanolic blank of a methylene chloride extract of the buffer. The Δ^4-3-ketosteroid concentrations were obtained from a standard cortisol curve and are expressed as µg equivalents of cortisol/100 mg tissue/2 hr of incubation. Recovery tests were run with 2 and 10 µg quantities of standard cortisol in 2.5 ml of buffer to check ef-

TABLE II. *In Vitro* Response of LAF_1 Mouse and WR Rat Adrenal Tumors to Low Dosages of ACTH.

	μg equiv. cortisol/100 mg/2 hr			
mU ACTH per 100 mg	LAF_1 3773 ♂	LAF_1 3680 ♂	LAF_1 3672 ♂	WR 274 ♀
.0	2.3	2.9	2.3	.7
.5	6.7	10.6		2.2
1.0	8.7	12.6	11.7	3.0
2.0	10.7	15.6	13.4	
2.5				10.0
3.0			14.4	
4.9				9.9
7.0				9.6
12.6				11.5
24.6				12.2

ficiency and precision of the extraction procedures. In 5 experiments at each concentration, the mean recoveries were 2.1 ± 0.2 μg and 9.8 ± 0.3 μg, respectively. When no peaks in absorption were observed at 242 mμ, the data were not included in the calculations. The steroidal composition of the ethanolic extracts was determined by paper chromatographic methods(4,5).

Results. Incubation studies. The mouse and rat adrenal tumor Δ^4-3-ketosteroid production with and without ACTH is recorded in Table I. The mouse adrenal tumors increased their secretion of steroids about 6 fold following addition of ACTH to the incubation medium. With the rat adrenal tumors, the increase was greater due to lower basal secretory capacity of the tumor slices in the absence of ACTH and higher secretory rates in the presence of ACTH. The steroid secretory rates of the tumor slices seemed to fall in the same range despite variations in tumor latency periods in the rat(1) following different types of operative treatment(Table I).

The sensitivity of these experimental adrenal tumors to ACTH was very high. In Table II, maximal steroid secretion of both rat and mouse adrenal tumor slices was obtained between 2 and 3 mU of ACTH per 100 mg tissue. These peak steroid values are similar to those in Table I where higher dosages of ACTH were given.

Incubation time studies of the total Δ^4-3-ketosteroid secretion of mouse and rat adrenal tumor slices are shown in Fig. 1.

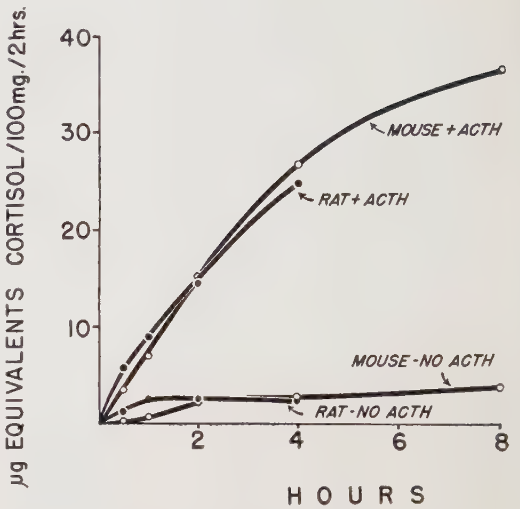

FIG. 1. Rates of Δ^4-3-ketosteroid secretion by mouse and rat adrenal tumor slices with maximal amounts of ACTH (60 mU for the mouse, 115 mU for the rat) and control slices.

The basal secretory rates of the normal mouse and rat adrenal segments (medulla intact) were higher per unit weight than those exhibited by the respective adrenal tumor slices (Tables III and IV). In turn, the maximal responses to ACTH by the normal tissues were also higher, resulting in activity ratios approximating that of the mouse adrenal tumor slices. Minimal stimulatory concentrations of ACTH for the normal mouse and rat adrenals were not determined. In testings with our group of normal rat adrenals, ACTH concentrations of 75-100 mU were maximal; with the normal mouse adrenals,

TABLE III. Response of Normal Male LAF_1 Mouse Adrenal Halves to ACTH.

	mU ACTH per 100 mg	μg equiv. cortisol/100 mg/2 hr*	Stimulation ratio†
	0	6.4	10.4
	336	66.5	
	0	14.4	4.1
	210	59.5	
	0	6.3	5.6
	640	35.3	
	0	6.9	
	630	70.0	10.1
	560	54.7	7.9
Avg	0	8.5	7.6
	210-640	57.2	

* One flask containing 10-11 adrenal pairs was used for each determination.

† See footnote Table I.

TABLE IV. Response of Normal Male WR Rat Adrenal Halves to ACTH.

mU ACTH per 100 mg	No. of[*] determinations	μg equiv. cortisol/100 mg/2 hr	Stimulation ratio[†]
0	2	18.4 (17.5–19.2)[‡]	4.9
100	2	90.5 (89.5–91.5)	
0	2	18.3 (18.2–18.3)	3.9
400	5	72.1 (62.5–87.5)	
0	3	9.1 (7.1–10.4)	5.6
600	3	50.5 (46.7–55.7)	
Avg 0		15.3	4.6
100–600		71.0	

[*] Each flask contained 1 pair of adrenals.
[†] See footnote Table I.
[‡] Avg and range.

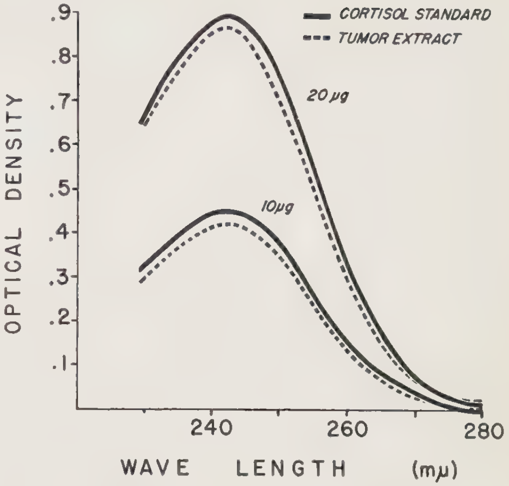

FIG. 2. Comparison of absorption spectra of standard cortisol in 95% ethanol and of methylene chloride extracts of adrenal tumor incubates redissolved in 95% ethanol.

the levels of ACTH used were assumed to be maximal.

In Fig. 2, the absorption curves of standard cortisol solutions are compared to experimental curves of adrenal tumor ethanolic extracts. Since the curves appeared to be identical in shape at similar peak readings, the presence of an interfering substance seems unlikely.

Chromatography. Under ACTH stimulation, the major steroid synthesized by normal mouse adrenal incubates was corticosterone (72% of the total Δ^4-3-ketosteroids), 11β-hydroxy-Δ^4-androstene-3,17-dione being present in small quantities (Table V). Adrenal tumor tissue, when incubated with ACTH, produced approximately equal amounts of these 2 steroids. However, 25-65% of the total Δ^4-3-ketosteroids in the tumor incubates

TABLE V. Corticosterone and 11β-Hydroxy-Δ^4-androstene-3,17-dione Content of Δ^4-3-Ketosteroids Extracted from Incubates of Normal Adrenals and Adrenal Tumor Tissue from LAF$_1$ Mice.

Exp.	mU ACTH per 100 mg	Condition of incubation	mg tissue	Δ^4-3-ketosteroids, μg/100 mg[‡]	Corticosterone μg/100 mg	%	11β-Hydroxy-Δ^4-androstene-3,17-dione μg/100 mg	%
			Normal mouse adrenals					
32, 38	0	Standard[†]	90	10.4	×		×	
”	275	”	80	63.0	45.0	72	8.7	14
			Mouse adrenal tumor					
22–24	0	Standard	1000	3.3	×		×	
22	1.5[*]	”	1100	13.2	2.6	20	2.0	15
23	1.5[*]	”	1200	8.6	1.6	19	2.3	27
24	2.0[*]	”	900	14.1	3.2	23	3.4	24
28	0	”	1100	2.5	×		.5	20
”	60	½ & 1 hr incub.	600	5.5	1.0	18	3.2	58
”	”	2 hr incub.	300	15.7	9.5	60	2.9	18
”	”	4 ” ”	200	27.2	12.1	45	7.1	26
”	”	8 ” ”	300	34.3	4.7	14	3.0	9
36, 37	25	No glucose in buffer	600	8.0	×		3.4	43
”	”	Standard	600	11.5	4.9	43	1.9	17

[*] Avg ACTH conc. in 3 flasks. Individual flasks contained .5, 1 and 2 mU ACTH (Exp. 22 and 23), and 1, 2 and 3 mU ACTH (Exp. 24).
[†] Standard incubation conditions as described in text.
[‡] Determined as "μg cortisol" by measuring O.D. at 242 mμ in Beckman model DU Spectrophotometer.
× = Not detected on paper chromatograms.

consisted of unidentified compounds. Analysis of 2 normal rat adrenal incubates for steroid content yielded 63% (11.4 μg/100 mg tissue without ACTH) and 39% (20.0 μg/100 mg tissue with ACTH) corticosterone in the total Δ^4-3-ketosteroid fraction. In 2 rat adrenal tumors incubated with ACTH, 5.7 μg and 2.3 μg of corticosterone and 2.0 μg and 2.6 μg per 100 mg tissue of a compound with chromatographic behavior of cortisone, respectively, were found. The sums of the 2 steroids represented 54% and 35%, respectively, of the total Δ^4-3-ketosteroids present in the tumor slice incubates, the remainder consisting of compounds yet to be identified.

The influence of glucose on steroid synthesis by the mouse adrenal tumor slices was investigated. The absence of glucose from the incubation medium had no effect on basal secretory rate, but the steroid secretion at maximal levels of ACTH with glucose was increased about 40% in 2 experiments as was in experiments of Schönbaum *et al.* using rat adrenal segments *in vitro*(6). The presence of glucose, however, effected a change in concentration of corticosterone and 11β-hydroxy-Δ^4-androstene-3,17-dione in a reciprocal manner (Table V, Exp. 36, 37).

Three adrenal tumors‡ originating in castrated ce strain mice and transplanted in the strain of origin were investigated for steroid secretion. At autopsy the tumor-bearing hosts (2 male and 1 female) showed signs of stimulation by gonadal hormones. Tumors 4-6 g in weight from the 3 strains were incubated with and without ACTH. No extractable Δ^4-3-ketosteroids were detected.

Discussion. The secretory response of the mouse and rat adrenal tumor slices to a low and narrow range of ACTH concentrations indicated a much greater sensitivity of adrenal tumor than normal adrenal tissue to the hormonal stimulation. Maximal stimulation of the normal rat adrenals (per unit weight) required about 30 times more ACTH than rat adrenal tumor. If log-dose response curves are drawn for the mouse values in Table II and the linear curves extrapolated to the base secretory levels, these tumor slices may well respond to 0.1 or 0.2 mU ACTH.

These adrenal tumors were able to secrete adrenocorticoids in the absence of ACTH. A theoretical comparison of the secretory potency of a 5 g tumor versus that of intact adrenals in the mouse and rat reveals some very striking results. A 5 g mouse adrenal tumor could theoretically secrete 130 μg of corticoids in 2 hours in the absence of ACTH and about 680 μg in 2 hours in the presence of maximal quantities of ACTH. On the other hand, a pair of normal mouse adrenals weighing about 4 mg would secrete less than 0.4 μg of corticoids in 2 hours in the absence of ACTH and about 2.3 μg in 2 hours in the presence of ACTH. Similar calculations comparing a 5 g rat adrenal tumor with a pair of rat adrenals (about 33 mg/pair) would show a secretion of 95 μg and 5 μg corticoids in the absence of ACTH and 870 μg and 25 μg corticoids per 2 hours with excess of ACTH, respectively. Thus, no endogenous ACTH would be necessary for the maintenance of hypercorticoidism in the tumor-bearing mouse or rat. Blood corticoid assays are being carried out to determine if the corticoid level is really as high as indicated by the tumor secretory potential.

The presence of corticosterone, 11-dehydrocorticosterone, cortisone and possibly 11β-hydroxy-Δ^4-androstene-3,17-dione, among other steroids, in rat adrenal effluents has been demonstrated(7,8,9). Corticosterone and 20α-dihydrocorticosterone have been identified in mouse serum(10). Under the experimental conditions reported here, the primary adrenal steroids synthesized by mice of the LAF_1 strain appear to be corticosterone and 11β-hydroxy-Δ^4-androstene-3,17-dione. In addition to these compounds, adrenal tumor incubates yielded substances suggestive in their chromatographic behavior of progesterone, 11β-hydroxyprogesterone, 11-dehydrocorticosterone and Δ^4-3-keto-20, 21-dihydroxy steroids. The quantitative (and possibly qualitative) differences in steroid production between normal mouse adrenals and adrenal tumor suggest different enzyme patterns and/or reaction kinetics and equilibria in the 2 types of tissues. The marked decrease in corticosterone accumulation in tumor tissue may be explained by a combination of reduced synthesis and increased adrenal metabolism of corticosterone to other steroids. The finding of Δ^4-3-ketosteroid with 21-desoxy or 20,21-dihydroxy side chains, and of 11-dehydrocorticosterone lend support to this hypothesis.

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‡ Kindly given to us by Dr. Arthur Kirschbaum.

The absence of any detectable amounts of 17α-hydroxylated corticosteroids in the mouse adrenal tumor or normal adrenal tissues confirms the *in vivo* study of Southcott(10) and the *in vitro* study of Hoffman(11).

Summary. 1) Slices from transplants of 2 secretory adrenocortical tumors were incubated with and without ACTH. Incubation mixtures were extracted and the total Δ^4-3-ketosteroids quantitatively determined from peak optical densities at 242 $m\mu$. Basal steroid secretion of the mouse and rat tumor slices averaged 2.6 and 1.9 μg equiv. cortisol/100 mg/2 hours respectively; with maximum stimulating amounts of ACTH, the average rates were 13.6 and 17.4 μg, respectively. Both tumors responded maximally to 2-3 mU of the hormone. 2) Under ACTH stimulation, corticosterone and 11β-hydroxy-Δ^4-androstene-3,17-dione were produced in approximately equal amounts by the mouse adrenal tumors. However, 25-65% of the total Δ^4-3-ketosteroids consisted of compounds still to be identified. The secretion of the rat adrenal tumor slices consisted mainly of corticosterone. 3) Glucose in the buffer stimulated the total steroid secretion of the mouse adrenal tumor slices about 40%. 4) An adrenal tumor that secreted gonadal hormones was incubated with and without ACTH. No measurable Δ^4-3-ketosteroids were detected in the incubates.

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Effect of Varying Levels of DL-Tryptophan in Diet on Dental Caries in Rats.[*] (23203)

Wolfgang Buttner[†] and Joseph C. Muhler
Indiana University, Department of Chemistry and School of Dentistry, Bloomington.

The chemistry of the salivary glands of the rat has recently received new interest due to experimental evidence indicating a correlation between activity of the thyroid gland, histologic picture of the salivary glands, and incidence of dental caries(1,2). Moreover, protein metabolism in the salivary glands as it relates to caries experience deserves special consideration, since proteolytic activity of rat salivary glands has been shown to be dependent upon normal thyroid function(3). Tryptophan is of particular interest in this relationship since "significantly greater amounts of tryptophan or tryptophan-like compounds in caries-free (human) salivas compared to caries-susceptible" has been reported(4).

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[†] Post-Doctorate Fellow. On leave of absence from University of Mainz, Germany.

TABLE I. DL-Tryptophan Contents of Various Diets Used, Salivary Tryptophan Levels and Incidence of Dental Caries in the Rat.

		Salivary tryptophan level			Dental caries experience		
Group	DL-Tryptophan added to diet (g/kg diet)	No. of samples	Conc. (mg %)	P	No. of animals	Mean No. of lesions	P
I	.6	24	32 ± 1.4*	.01	24	8.1 ± .5*	.3
II	1.8	28	42 ± 2.8	.001	28	8.0 ± .6	.4
III	5.4	25	48 ± 2.1	"	25	9.1 ± .5	.04
IV		27	25 ± 1.1		27	7.4 ± .5	

* Stand. dev.

Therefore, it is of interest to determine experimentally if DL-tryptophan possesses any anticariogenic properties if fed to rats receiving a cariogenic diet.

Materials and methods. A total of 120 weanling Sprague-Dawley strain rats were divided equally into 4 groups according to sex and initial body weight. Groups 1 through 3 received a cariogenic corn diet to which were added varying amounts of DL-tryptophan (Table I). Group 4 received the same diet without added tryptophan and served as a control. Composition of the cariogenic diet in percentage was as follows: yellow corn grits, 52.7; ground yellow corn, 11.3; powdered whole milk, 30.0; alfalfa, 4; iodized sodium chloride, 1.0; and irradiated yeast, 0.2. The animals were housed in pairs in raised screen cages in an air-conditioned room, and all received fluorine-low drinking water ($F = 0.05$ μg/ml) *ad libitum.* After 110 experimental days, saliva of each rat from each group was collected and analysed for tryptophan. Sodium nembutal was used for anesthesia (0.53 ml of a 0.5% stock solution of sodium nembutal per 100 g body weight was injected intraperitoneally). Pilocarpine hydrochloride (10 mg/g body weight injected intraperitoneally) was used to increase salivary flow. By such procedures, approximately 2 ml of saliva could be collected conveniently within 10 minutes. The method of tryptophan analysis(5,6) was adapted to salivary tryptophan analysis. 7 ml of distilled water was added to 1.0 ml of saliva. Following thorough mixing, 2 ml of the solution were transferred to a test tube, and 0.5 ml of glyoxylic acid(7), and 0.5 ml of 0.01 M copper sulfate solution were added. The test tube was placed in an ice water bath and 8.0 ml of a sulfuric acid solution (60 parts H_2SO_4 to 40 parts distilled water) were slowly added. After 10 minutes, the sample was placed into a boiling water bath for 3 minutes, then cooled at room temperature for 1 hour. The amount of salivary tryptophan was determined with the aid of a Klett-Summerson photoelectric colorimeter by comparison to a standard curve obtained by running known amounts of DL-tryptophan through the procedure. A 540 mμ filter was used. After 120 days, the animals were sacrificed by ether and the heads were removed for dental caries evaluation as previously described(8).

Results. The effect of tryptophan on incidence of dental caries and on salivary tryptophan level is shown in Table I. The effect of adding 0.6 or 1.8 g/kilo DL-tryptophan to the stock corn cariogenic diet was associated with a small and non-significant increase in incidence of dental caries when compared to controls receiving the same diet without added tryptophan. When 5.4 g/kilo tryptophan was added to the diet, there was a significant increase in incidence of dental caries.

Determinations of salivary tryptophan levels indicate an increasing amount of tryptophan with increasing dietary tryptophan concentration. It is not possible by this method of analysis to distinguish between free tryptophan or tryptophan bound in some simple form, but since there seems to be no correlation to salivary tryptophan level and dental caries in the rat, it does not seem important to design experiments in order to clarify this point. The failure of DL-tryptophan to reduce incidence of dental caries in the rat even though there was a significantly greater salivary tryptophan level suggests that the higher salivary tryptophan level observed in caries-immune humans(4) is not the only factor involved in caries immunity in humans and that a similar immunity to caries cannot be induced in rats by feeding tryptophan.

The daily weight gains in the animals receiving the tryptophan were somewhat less. However, these differences were insignificant in all groups except in the one receiving 5.4 g tryptophan per kg of diet. In this group the final weight of both the males and the females was about 20% less than the controls.

The role of protein degradation in the oral cavity or within the salivary glands themselves must bear some relationship to caries immunity in the rat, since the salivary gland-thyroid studies clearly indicate an increased proteolytic effect of the salivary gland homogenates following administration of desiccated thyroid, while a marked decrease resulted following administration of I^{131} or thiouracil. The latter substances are known to be related to caries immunity or susceptibility, respectively. The anatomical structures within the salivary gland affected by thyroid activity are the serous tubules, and Shafer[‡] has noted that following thyroxine administration large numbers of the granules pass into the excretory ducts of the salivary glands. Since these granules are thought to be predominantly protein in nature, the significance of proteolytic enzymes, protein metabolism, and the role of different amino acids on caries resistance or susceptibility in the rat needs much further study.

Summary. Dental caries experience in rats was studied following feeding of varying levels of DL-tryptophan as a constituent of a cariogenic diet. When either 0.6 or 1.8 g/kilo tryptophan was added to the diet there was an increase in dental caries, although the differences were not significant. When the diet contained 5.4 g/kilo tryptophan there was a significant increase in incidence of dental caries. Concomitant with the increased dietary tryptophan level, there was an increased salivary tryptophan concentration.

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[‡] Personal communication.

Status of Deoxycorticosterone as Intermediary in the Biosynthesis of Cortisol.* (23204)

John Eichhorn[†] and Oscar Hechter

The Worcester Foundation for Experimental Biology, Shrewsbury, Mass., and Department of Physiology, Boston University School of Medicine, Boston, Mass.

The conversion of cholesterol to 17α-hydroxycorticosterone (cortisol) and corticosterone, demonstrated in adrenal preparations (1,1a,2,3) has been postulated to proceed via the reaction sequence shown in Fig. 1(1,4). All of the individual reactions shown have been definitively demonstrated in cell-free systems, and the available *in vivo* data are consistent with this scheme(4). The postulation of an orderly series of hydroxylations of the progesterone molecule was derived in large part

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[†] Portion of thesis submitted in partial fulfillment of requirements for Doctor of Philosophy degree, Dept. of Physiology, Boston University, Mass.

$$\begin{tikzcd}
\text{Cholesterol} \arrow[r] & \text{Pregnenolone} \arrow[r] & \text{Progesterone} \arrow[r] \arrow[d] & \text{DOC} \arrow[r] & \text{Corticosterone} \\
 & & \text{17-OH progesterone} \arrow[r] & \text{17-OH DOC} \arrow[r] & \text{Cortisol}
\end{tikzcd}$$

FIG. 1. Corticosteroidogenic sequence.

from the bovine perfusion findings that while deoxycorticosterone (DOC) was converted to corticosterone, neither DOC nor corticosterone could be further hydroxylated at C-17α to form cortisol; subsequent studies, using homogenates from bovine and porcine adrenals, yielded essentially similar results(5,6,7,8). Recently, however, it has been reported by Heard and his associates(2,3) that C^{14}-DOC is readily converted in good yield to cortisol as well as corticosterone in adrenal preparations from a variety of species including bovine. Heard *et al.* have therefore concluded that the sequence of hydroxylations postulated in Fig. 1 is not obligatory and that alternative routes are operative to a major degree. To clarify the apparent discrepancy between the work of Heard's group and the previous findings cited, the role of DOC as an intermediary has been reinvestigated in cow adrenal homogenates which form corticoids from endogenous precursors. For this purpose tracer amounts of DOC-21-C^{14} and progesterone-4-C^{14} were incubated with homogenates prepared in various media, and the degree of incorporation into the cortisol and corticosterone formed was measured. In all homogenates studied, it was observed that while DOC-21-C^{14} was incorporated into the corticosterone, no incorporation into the cortisol formed in the system could be detected; progesterone-4-C^{14}, on the other hand, was incorporated into both cortisol and corticosterone. While DOC-21-C^{14} was not converted to cortisol, it did give rise to certain unknown C^{14}-products, of polarity similar to but not identical with cortisol, which could be separated from cortisol by careful paper chromatographic fractionation.

Methods. Breis of decapsulated adrenocortical tissue from fresh cow glands were homogenized in each of the following media using a loose-fitting Potter-Elvehjem glass apparatus (4 ml medium per g tissue): (a) 0.025*M* sucrose, (b) 0.154*M* KCl and (c) 0.154*M* NaCl. All homogenizing media were buffered with M/150 Sorensen's phosphate buffer and supplemented with niacinamide, sodium fumarate and $MgSO_4$ (all at a concentration of 5 mM/l). After homogenization ATP‡ and DPN§ were added to a final concentration of 1.0 and 0.5 mM/l, respectively. All operations involved in the preparation of these homogenates were carried out near 0°. Homogenate aliquots corresponding to 35 to 100 g tissue were added to reaction vessels containing 298 to 600 μg of progesterone-4-C^{14} (specific activity (S.A.) 1.60×10^6 cts/m/mg) or 332 to 700 μg DOC-21-C^{14} (S. A. 1.55×10^6 cts/m/mg) suspended in about 0.3 ml of propylene glycol, and then incubated for 2 hours at 38° with shaking (gas phase—100% O_2). Aliquots of the same homogenate extracted without incubation served to measure the endogenous cortisol and corticosterone initially present. The C^{14}-progesterone sample used in these experiments was radiochemically homogenous in the ligroin-propylene glycol paper chromatography system(9), and had been further chromatographed on silica gel; after prolonged percolation, benzene-ethyl acetate (20:1) eluted C^{14}-progesterone from the column as a sharp symmetrical peak of radioactivity. The C^{14}-DOC sample used, obtained as the 21-monoacetate, was first purified as the acetate using the toluene-hexane(1:1) - propylene glycol paper chromatography system; when homogenous in this system, the acetate was saponified and the free DOC was chromatographed in the toluene-propylene glycol system until it was radiochemically homogeneous in this system.

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‡ ATP—Adenosine triphosphate

§ DPN—Diphosphopyridine nucleotide

TABLE I. Products Isolated from Bovine Adrenocortical Homogenates Prepared in Various Media and Incubated with C^{14}-DOC and C^{14}-Progesterone.

	Substrate*	Substrate added (μg)	Tissue wet wt (g)	Medium	Net steroid production (mg/100 g/2 hr)		C^{14}-Substrate converted (μg/100 g/2 hr)	
					Cortisol	Corticosterone	Cortisol	Corticosterone
A	Progesterone-4-C^{14}	322	35	Sucrose	.89	1.31	18	51
				KCl	.82	.68	3	34
				NaCl	.77	1.18	4	81
	DOC-21-C^{14}	332	”	Sucrose	.76	1.98	$<.5$	137
				KCl	1.09	1.04	$<.5$	45
				NaCl	.82	1.02	$<.5$	72
B	Progesterone-4-C^{14}	298	60	Sucrose	2.58	2.60	52	123
		540	100	”	1.52	2.12	27	76
		600	46	KCl	1.16	2.09	10	182
	DOC-21-C^{14}	700	44	Sucrose	.76	2.50	$<.5$	415
		700	46	KCl	1.20	1.52	$<.5$	145

* S.A.—Progesterone, 1.60×10^6; DOC, 1.55×10^6 cts/min./mg.

Finally, the DOC was chromatographed on silica gel; after percolation of large volumes of a benzene-ethyl acetate mixture (10:1) the DOC was eluted by this solvent mixture as a sharp symmetrical peak. Corticosteroids were obtained from the homogenates by exhaustive extraction with acetone and ethyl acetate; the total extracts were combined and distilled *in vacuo*. The resulting aqueous solution was then thoroughly extracted with ethyl acetate. The lipoid extracts thus derived contained 90-95% of the added radioactivity. After partial removal of phospholipids in the crude extract by acetone precipitation in the presence of $MgCl_2$ (in the cold), the extract was further purified by silica gel chromatography. After eluting nonradioactive materials with hexane, benzene and benzene-ethyl acetate mixtures (9:1), about 75% of the radioactive materials and all of the cortisol and corticosterone present were eluted with a benzene-ethyl acetate mixture (1:1) and with ethyl acetate. The additional radioactivity eluted by methanol (about 25%) did not contain cortisol or corticosterone as determined by paper chromatography. The cortisol and corticosterone present in the silica gel eluates were isolated by procedures previously described(1), wherein the chloroform-formamide and toluene-propylene glycol systems of paper chromatography(9) were utilized for cortisol and corticosterone respectively. The cortisol and corticosterone zones located by ultraviolet scanning and characteristic spotting reagents (TPTZ, Bush soda fluorescence and H_2SO_4 fluorescence tests), were then eluted and rechromatographed in the same systems. The rechromatographed free steroid alcohols were then eluted, acetylated and chromatographed on toluene-propylene glycol (18 hr) and toluene-ligroin (1:1)/propylene glycol (8 hr) for cortisol acetate and corticosterone acetate respectively. The corticosteroid acetates were located by the reagents mentioned previously and then rechromatographed in the same systems. At this stage of purification the specific activities of the corticosterone acetate and cortisol acetate were determined by counting an aliquot (infinite thinness) in a flow-gas counter and determining the corticosteroid content with a modified Mader-Buck procedure using blue tetrazolium. During the entire course of purification, all chromatograms were scanned in a calibrated counting chamber, so that it was possible to locate zones of radioactivity. With the exception of the cortisol acetate zones derived from the DOC incubations, all of the above-mentioned zones contained a peak of radioactivity.

Results. The results obtained in these experiments are shown in Table I. In A, the various homogenates were all derived from the same pool of adrenocortical brei, incubated with both C^{14}-progesterone and C^{14}-

TABLE II. Values at Successive Stages of Purification (Sucrose Homogenate).

Substrate	Specific activity (counts/min./μg)		
	"Cortisol"	Cortisol acetate*	Cortisol acetate†
C^{14}-Progesterone	27	33	38
C^{14}-DOC	63	8	<0.8

* After first chromatogram. † 2nd chromatogram.

DOC and worked up simultaneously. In B, the results shown were obtained in individual experiments using different adrenal tissue. The results in both types of experiment are comparable in that: (a) in none of the homogenates was it possible to detect the conversion of C^{14}-DOC to cortisol, despite the fact that endogenous production of cortisol was clearly evident in all cases; (b) the conversion of C^{14}-DOC to corticosterone proceeds in all homogenates and (c) the conversion of C^{14}-progesterone to both cortisol and corticosterone is demonstrated. These results are fully consistent with the scheme postulated in Fig. 1. The marked effect of the ionic media upon the lesser degree of incorporation of C^{14}-progesterone into cortisol relative to corticosterone will be the subject of a subsequent paper.

While the cortisol finally isolated from the C^{14}-DOC incubation products did not contain detectable radioactivity, considerable radioactivity was observed in the cortisol zone prior to acetylation. Table II shows the changes in S.A. of the "cortisol zone" derived from C^{14}-DOC incubations in a typical sample at various stages of purification by paper chromatography; for comparison the results obtained with the "cortisol" derived from a C^{14}-progesterone incubation are also shown. It will be seen that with the material from C^{14}-DOC incubation a considerable amount of radioactivity is initially associated with the cortisol zone, and that with successive purification by paper chromatography the radioactivity disappears. In marked contrast, the radioactivity of the cortisol zone derived from C^{14}-progesterone remains relatively constant during these same fractionation procedures. While the present homogenate studies are not directly comparable to those of Heard *et al.* (2,3), the present findings raise the strong possibility that in the studies of Heard *et al.* C^{14}-DOC was converted to a radioactive product of mobility similar to cortisol, *which is not cortisol.*

In accord with our conclusion, recent work with rat adrenal tissue(11) has likewise failed to detect the conversion of DOC to cortisol, although it was reported that this steroid was converted to Porter-Silber reactive chromogenic material (presumably containing the dihydroxyacetone side chain characteristic of cortisol) and to a ketolic steroid zone of polarity intermediate between cortisone and corticosterone. Similarly, it has been reported that corticosterone is converted to more polar products, the nature of which has not been defined(10). Thus it seems clear that DOC can be converted in appropriate *in vitro* systems to products which are more polar than corticosterone as well as to corticosterone. However, until it has been shown, using unequivocal technics for the demonstration of radiochemical homogeneity, that DOC is actually converted to cortisol, there would appear to be no need to postulate an alternative pathway of cortisol production via DOC.

Summary. Homogenates of adrenocortical tissue which synthesize cortisol and corticosterone from endogenous precursors were prepared in various media and incubated with trace amounts of DOC-21-C^{14} and progesterone-4-C^{14}. While C^{14}-DOC was converted to corticosterone, and C^{14}-progesterone to both corticosterone and cortisol, C^{14}-DOC was not converted to cortisol. DOC, however, was converted to a C^{14}-product possessing a chromatographic mobility similar to cortisol, which was separated from cortisol by careful paper chromatographic fractionation.

The able assistance of Mrs. Ella Scully and Mr. Morris Feinstein is gratefully acknowledged.

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Meso-Inositol Requirement of HeLa and Human Conjunctival Cells.* (23205)

ROBERT P. GEYER AND R. SHIHMAN CHANG (Introduced by John C. Snyder)
Departments of Nutrition and Microbiology, Harvard School of Public Health, Boston, Mass.

A number of different human cells have been shown to require meso-inositol when grown *in vitro*(1). This is of considerable interest since it represents the first conclusive demonstration that meso-inositol is an essential nutrient for human cells. The report of Eagle *et al.*(1) was also of interest because no conclusive evidence was obtained that inositol was required by the cell strain derived from a human carcinoma, the HeLa cell.

In similar studies with the human conjunctival cells(2), regular success was obtained only after a greater concentration of inositol was used than the 1 μM suggested by Eagle *et al.*(1). In view of this, the requirement for inositol by the HeLa cell was also studied. These studies form the basis of the present report.

Methods. Human conjunctival cells(2) and HeLa cells(3) were used throughout these experiments. Stock cultures were propagated in 250 ml serum dilution, neutral glass bottles containing 10 ml of Eagle's basal medium(4) plus 10 to 20% human serum. Only stock bottles which showed the presence of a continuous sheet of polyhedral cells with clear cytoplasm and occasional mitoses were used. The actual experiments were conducted using sets of 4 roller tubes, 18 x 150 mm, containing a total volume of 1 ml of Eagle's medium plus 20% dialyzed human serum. Following inoculation, the tubes were slanted at a 2-5° inclination at 36°C for 16 to 20 hours and then were placed in a roller drum also at 36°C. The medium was replaced in all tubes on the third and fifth days. The cells were examined daily under 100x magnification. On the seventh day, the cells were trypsinized and counted with a Levy counting chamber (5). Dialysis of serum was accomplished as follows: 15 ml of serum was dialyzed in cellulose dialysis tubing[†] against 15 ml of Eagle's basal medium(4) for 24 hours; to maintain the pH at 7.2 to 7.4, the serum was dialyzed in a stoppered Erlenmeyer flask of 125 ml capacity. The dialysate was frozen at –80°C until used. The serum was dialyzed further against 4 changes of 0.85% NaCl, of about 1 liter each, for a total of 48 hours, and then against approximately 100 ml of Eagle's basal medium for one to 2 hours longer. Each dialysis procedure was carried out at 0 to 2°C with constant agitation. The volume of the dialyzed serum was routinely measured and usually showed an increase of 0.5 to 1.0 ml. The dialysate was routinely checked for serum protein contamination by the addition of

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[†] The Visking Corp.

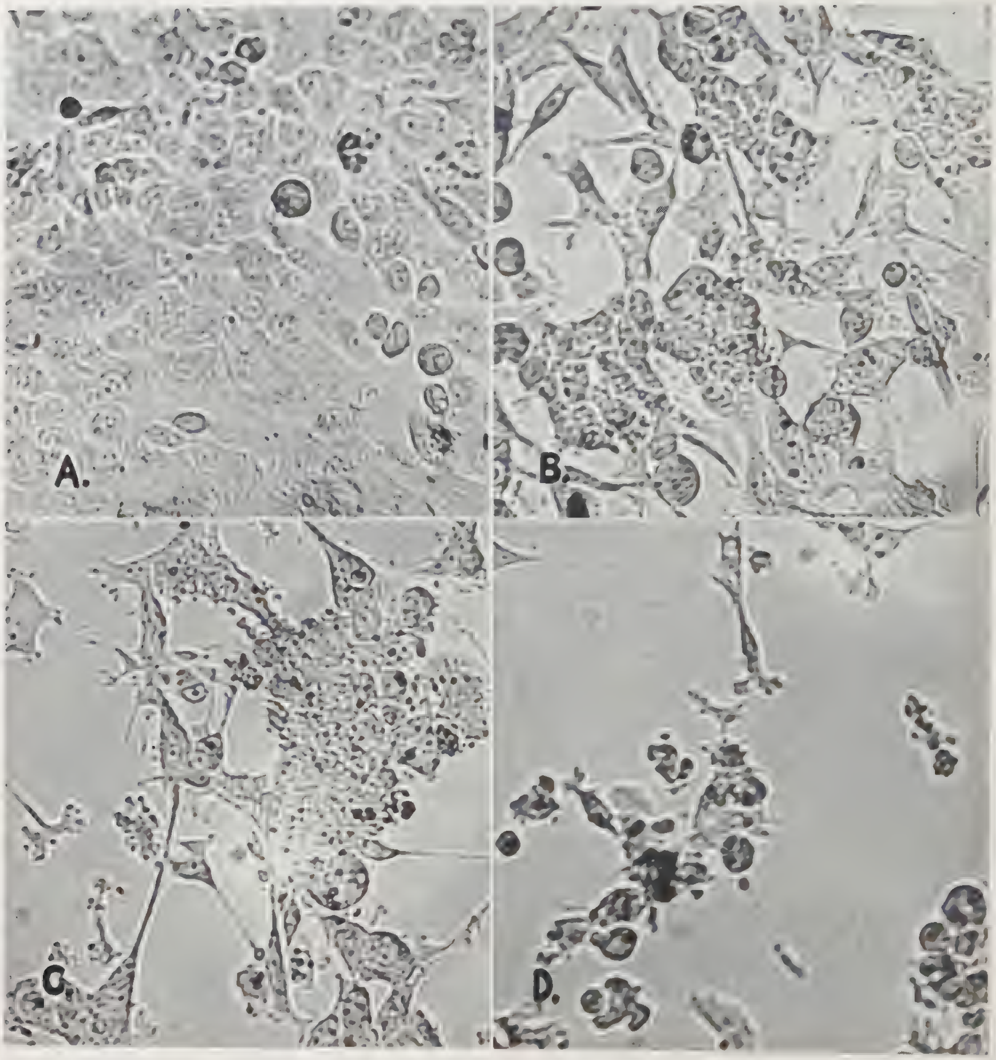

FIG. 1. Appearance of HeLa cells in medium without inositol. A. 3rd day. B. 5th day. C. 6th day. D. 7th day. (Magnification 100X.)

0.1 ml of 10% trichloracetic acid to 1 ml of the dialysate; the formation of turbidity was taken as indicative of the presence of proteins in the dialysate, and the dialysate was discarded. The dialyzed serum and dialysate were used at a final concentration equivalent to 20% whole serum. Meso-inositol[‡] was used in the experimental tubes at concentrations from 0.27 μM to 16 μM. Pertinent data and results are given in the appropriate table.

Results. In dialyzed serum diluted with Eagle basal medium, both the conjunctival and the HeLa cells showed characteristic progressive degenerative changes beginning on the fourth to sixth day. Such changes are illustrated in Fig. 1. Meso-inositol at a concentration of 1 μM did not prevent degenerative changes with 5 of the 6 sera tested in 5 separate experiments. Representative results are shown in Table I. By increasing the inositol concentration to 4 μM, degenerative changes were completely prevented with all sera tested. Further increase to 16 μM did

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[‡] Corn Products Refining Co.

TABLE I. Effect of Meso-Inositol on Multiplication of Human Conjunctival Cells *In Vitro*.*

Exp.†	Donor	Supplement‡	Avg No. of cells × 10^3/tube
1	Human A	None	0 (3+)‖
		Dialysate	106 ± 8 (0)
		Inositol§	19 ± 6 (2+)
2	Human B	None	0 (3+)
		Dialysate	214 ± 22 (0)
		Inositol	105 ± 25 (+)
	Horse L	None	5 ± 3 (3+)
		Dialysate	150 ± 24 (0)
		Inositol	65 ± 17 (+)
3	Human C	None	0 (3+)
		Dialysate	128 ± 19 (0)
		Inositol	182 ± 14 (0)

* These experiments show extremes when using different sera. A total of 5 experiments have been done using 6 different sera.

† Inoculi: 6,800, 10,000 and 12,000 cells/tube for Exp. 1, 2 and 3 respectively.

‡ Basal medium contained dialyzed serum at final conc. of 20% in Eagle's medium.

§ Meso-inositol used at final conc. of 1 μM.

‖ Morphological appearance of cells on 7th day: (0) = No degeneration, (+) = moderate, (2+) = advanced, (3+) = complete degeneration.

not significantly alter the multiplication rate. These results are presented in Table II. Thus the report of Eagle *et al.*(1) that meso-inositol is an essential nutrient for the conjunctival cells has been confirmed.

The HeLa cells, like the conjunctival cells, required inositol for rapid multiplication (Table III, Fig. 1). It is interesting that 1 μM inositol did not prevent degeneration of the HeLa cell in dialyzed human serum A and horse serum L diluted with Eagle basal medium as shown by results of Experiment 1 in Table III. However, when the experiment was repeated, satisfactory multiplication without grossly visible degeneration did occur (Exp. 3 in Table III). Such quantitative variations occurring in similar experiments have been frequently observed(6). This is probably caused by a difference in the physiological state of the initial cell inocula. In this instance, the amount of inositol carried along with the inoculum may be responsible. At any rate, in the present studies the HeLa cells invariably showed marked degeneration after seven days in media without inositol.

The reasons for the differences between the results of Eagle(1) and those reported here are not clear. The sera used in these studies were routinely dialyzed for 2 to 4 days to insure adequate removal of dialyzable factors. There are, however, 2 extraneous sources of inositol which may be of importance in obtaining non-consistent results either between experiments or between various sera. One of these is inositol carried along in the cell inoculum, since the stock medium contains adequate inositol. The other is inositol which was not completely removed by the dialysis procedure. Such inositol may be either in the form of phosphoric esters or inositides. The latter would be of especial importance for several reasons. First, phosphatides being colloids in aqueous solutions dialyze with difficulty(7). Second, phosphatides occur bound to serum protein(8). Third, McKibbin and Brewer(9) have reported that human plasma contains 74 μmoles of lipid inositol per liter. This concentration is sufficiently high so that sufficient inositol could be retained during dialysis due to the first and second factors. In this regard it is of interest that dialysis does not cause degradation of serum lipopro-

TABLE II. Effect of Varying Concentration of Meso-Inositol on Multiplication of Conjunctival Cells.

		Avg No. of cells × 10^3/tube at following concentrations of inositol					
Exp.*	Medium†	0	.25 μM	1 μM	4 μM	16 μM	Dialysate 20%
1	Human A	0 (3+)‡	2 (3+)	116 ± 40 (+)	294 ± 62 (0)	262 ± 31 (0)	238 ± 45 (0)
1	" Hu	0 (")	5 (")	27 ± 9 (+)	117 ± 50 (0)	130 ± 53 (0)	352 ± 46 (0)
2	" Ha	0 (")		220 ± 43 (0)	296 ± 49 (0)		132 ± 41 (0)

* Inoculi: 10,000 and 20,000 cells/tube in Exp. 1 and 2 respectively.

† Eagle's basal medium containing 20% dialyzed serum from the specified donor and meso-inositol or serum dialysate at the specified concentrations.

‡ Morphological appearance of cells on seventh day: (0) = No degeneration, (+) = moderate degeneration, (2+) = advanced degeneration, (3+) = complete degeneration.

TABLE III. Effect of Meso-Inositol on Multiplication of HeLa Cells.

		Avg No. of cells × 10^3/tube at following concentrations of inositol					
Exp.*	Medium†	0	.25 μM	1 μM	4 μM	16 μM	Dialysate 20%
1	Human A	0 (3+)‡		11 ± 6 (2+)			103 ± 69 (0)
	Horse L	2 (")		60 ± 13 (+)			203 ± 40 (0)
2	Human W	0 (")					101 ± 17 (0)
3	Human A	22 ± 2 (3+)	50 ± 9 (2+)	272 ± 28 (0)	280 ± 20 (0)	284 ± 32 (0)	217 ± 43 (0)
	Horse L	35 ± 17 (3+)	131 ± 27 (+)	275 ± 36 (0)	292 ± 20 (0)	191 ± 13 (0)	281 ± 31 (0)

* Inoculi: 15,000, 13,000 and 27,700 cells/tube in Exp. 1, 2 and 3 respectively.
† & ‡ See footnotes to Table II.

teins(10).§ The extent to which complex forms of inositol are utilized by human cells is a matter for future study.

Summary. A concentration of meso-inositol of at least 4 μM was necessary for maximum multiplication of human conjunctival cells grown *in vitro*. Inositol was found to be essential for multiplication of HeLa cells.

The authors are grateful to Dr. John C. Snyder for his suggestions and interest in this work.

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§ In our experiments analysis of human and horse sera before and after 48 hours of dialysis showed little alteration in cholesterol content.

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Lipid Mobilizer (LM) from Posterior Pituitary of Hogs.* (23206)

JOSEPH SEIFTER AND DAVID H. BAEDER
Wyeth Institute for Medical Research, Radnor, Pa.

Injection of plasma or plasma dialyzate, obtained from animals administered cortisone or subjected to stress, induced hyperlipemia in intact, adrenalectomized or hypophysectomized rats previously fasted and sensitized by exposure to potential hepatotoxins(1-4). The dialyzate and highly potent material crystallized from it differed from other hyperlipemia inducing agents in not requiring the presence of either the adrenals or pituitary for its action(2-4). These studies suggested that LM was released from the pituitary gland. The data in this paper establish the presence of a

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TABLE I Effect 1 mg Dialyzate of Hog Posterior Pituitary Lobes/kg IV on Plasma Lipid Components of Various Species.

	Time in hours post injection																	
	0			1			2			4			6			24		
Species	CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP
Intact rats (10/hr)	62	104	5	196	212	9	262	303	10	302	353	12	348	392	12	184	212	8
Hypophysectomized rats (20/hr)	58	92	5				242	303	11									
Adrenalectomized rats (20/hr)	64	88	5				258	312	11									
Mice (10/hr)	52	95	5	195	208	8	312	340	12	402	386	15	440	490	10	120	212	8
Guinea pigs (10/hr)	85	102	5	186	195	9	245	260	10	322	330	11	385	402	13	84	92	6
Rabbits (12/sample)	62	90	5	190	262	9	240	304	11	320	386	13	360	412	14	75	142	8
Dogs (6/sample)	120	160	6	290	340	11	380	400	18	412	400	19	402	440	18	196	220	9

CH = Total plasma cholesterol; FA = Total fatty acids; LP = Lipid phosphorus. All values in mg %.

lipid mobilizing factor in the posterior pituitary of hogs.

Materials and methods. Lyophilized anterior and posterior lobes from hogs were prepared by Armour Laboratories. Duplicate preliminary studies demonstrated that LM was present in physiological salt solution (PSS) extracts of posterior pituitary but not in those of anterior pituitary or rat gastrocnemius muscle. A lot of posterior pituitary extract was then prepared as follows utilizing conditions that assured absence of bacterial and pyrogen contamination. Ten g finely diced lyophilized posterior pituitary was macerated with 3 g alcohol washed sea sand and addition of 100 ml PSS in small aliquots. The suspension was centrifuged 2 hours (5°C) at 4000 rpm and the withdrawn supernatant diluted to 500 ml with PSS was passed through an ultra-fine porcelain bacteriological filter (5°C). 100 ml of filtrate was dialyzed in a sealed container through Viscose No-Jax® casing against 400 ml deionized water for 24 hours at 5°C and the dialyzate was freeze dried for 18 hours. A 0.1% solution of the dry material (Wy-1323) was filtered bacteriologically and was sterile and non-pyrogenic. All animals were sensitized, fasted, injected and bled for lipid analyses as previously described(2-4). Adrenalectomized rats were maintained on 0.9% NaCl in the drinking water, and hypophysectomized rats received supplements of oranges and white bread. Operated animals were used 10-14 days after surgery.

Results: Injection of Pitressin®, Pitocin®, muscle extract, anterior lobe extract, and posterior lobe extract into groups of 20 hypophysectomized rats resulted in hyperlipemia only in the group administered posterior lobe extract. Dialyzate (0.65 mg) equivalent to 5 mg of posterior lobe produced at least a 2-fold increase in total plasma cholesterol, total fatty acids and lipid phosphorus in 60 intact rats, 20 hypophysectomized rats, 60 mice, 60 guinea pigs, 12 rabbits and 6 dogs. The hyperlipemia action of 1 mg of dialyzate/kg in a variety of species is demonstrated in Table I. The dose response curve obtained in 40 intact rats with another batch of dialyzate established a potency of

0.2 mg of dialyzate/kg to produce a 2-fold elevation of plasma cholesterol.

Discussion. Our previous work established that the pituitary was necessary for release of LM. The data presented in this paper establish that hog posterior pituitary dialyzate contained LM activity comparable to that of plasma dialyzate. Other points of similarity between plasma LM and posterior pituitary LM were observed in groups of 10 rats administered 1 mg/dialyzate/kg. The hyperlipemia occurring in either group 45 minutes later was not abolished by administration of 400 U of heparin/kg. *In vitro* anti-clearing activity of pituitary LM was identical with the activity previously reported for plasma LM(1,2). Exact identity of pituitary LM with plasma LM remains to be established.

Summary and conclusions. 1. Dialyzate of hog posterior pituitary lobe induced hyperlipemia in rats, mice, guinea pigs, dogs and rabbits. 2. The LM activity was indistinguishable from that of plasma dialyzate from cortisonized animals. 3. Neither Pitressin® nor Pitocin® had LM activity. 4. Hog anterior pituitary extracts had no LM activity. 5. LM appears to be a hitherto undescribed hormone either elaborated or stored in the posterior pituitary.

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Inhibition of Histamine-Stimulated Secretion by Topical Application of Antihistaminics to the Canine Mucosa.* (23207)

HENRY D. JANOWITZ AND FRANKLIN HOLLANDER
(With the technical assistance of Sylvan Douglis)
Gastroenterology Research Laboratory, Mount Sinai Hospital, N. Y. City.

Failure of antihistaminics to inhibit histamine-stimulated gastric acid secretion is generally accepted. The extensive literature supporting this generalization has been carefully summarized by Robertson and Grossman(1). The gastric receptors for histamine, therefore, appear to present a puzzling exception to the almost universal blocking action of antihistaminics on histamine receptors in other organs. These authors, however, also have collected some fragments of evidence which indicate that high concentrations of antihistaminic compounds, present locally in the gastric mucosa, may block its secretory response to histamine. Wood(2) was able to block histamine-stimulated acid secretion in anesthetized cats by retrograde injection of phenergan, neoantergan, and antistin into the ligated splenic artery, so that these compounds reached the gastric mucosa in high concentration. A chemical analogue of phenergan without antihistaminic activity (R.P. 3300) was without effect. In their own experiments, Robertson and Grossman irrigated canine pouches with high concentrations of several antihistaminics (25 and 50 mg/ml of neoantergan; 6.25, 12.5, and 25 mg/ml of pyribenzamine; 12.5 and 25 mg/ml of benadryl; and 50 mg/ml each of trimeton and chlortrimeton) and thereby inhibited the secretion of acid stimulated by histamine. However, these authors believed that, strictly speaking, their results were not "antihistaminic" but rather a direct irritant effect on the gastric glands, since 1) these compounds inhibited acid secretion induced by the parasympathomimetic drug, urecholine, as well; and 2) these compounds stimulated flow of a mucoid liquid from the pouches, which was frequently bloody. The concentrations of drug which

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TABLE I. Effect of Antihistaminics, Applied Topically to Gastric Mucosa of Canine Heidenhain Pouches, on Histamine-Stimulated Gastric Acid Secretion.

Compound	Conc. (mg/ml)	Output of HCl (mEq)			% inhibition (mean % decrease)
		Control	Test	% decrease	
Isotonic saline		2.23	1.94	13	
		1.17	1.11	5	
		2.26	2.26	0	
		1.94	.69	13	
		1.96	2.15	10	
		4.10	2.58	37	
		1.40	1.57	12	
		1.50	1.47	2	
		4.16	4.23	2	
		3.09	3.30	7	10
Phenergan hydrochloride	2	2.45	.84	66	
		1.04	.08	92	
		3.26	.47	86	
		3.39	2.41	29	
		3.97	.73	82	
		2.52	.31	88	
		.27	.00	100	
		.57	.04	93	
		1.79	.62	65	
		1.55	.45	71	
		.30	.00	100	80
	4	.58	.00	100	
		.52	.00	100	
		.97	.00	100	
		3.03	.00	100	
		2.08	.00	100	
		1.48	.00	100	100
Phenergan methosulfate	2	.60	.43	28	
		2.18	1.02	53	
		.91	.42	54	
		.55	.40	27	41
	4	1.97	.71	64	
		1.43	.70	51	
		1.62	.88	46	
		.88	.40	55	
		.63	.33	48	
		2.88	1.22	58	54
Pyribenzamine	2	3.25	2.67	18	
		3.18	1.18	63	41
	4	2.76	1.98	28	
		3.38	1.45	57	
		2.75	1.54	44	
		.72	.24	67	
		1.75	.16	91	57
	6	1.48	.26	82	
		.49	.11	79	
		2.12	.83	61	74
	8	2.02	.72	64	
		2.10	.34	84	
		.61	.08	87	
		1.78	.50	72	77
Neoantergan	8	.75	.19	75	
		.36	.19	47	61
	10	1.99	.77	61	
		1.28	.40	69	65

TABLE II. Effect of Antihistaminics, Applied Topically to Gastric Mucosa of Canine Heidenhain Pouches, on Urecholine-Stimulated Gastric Acid Secretion.

		Output of HCl (mEq)			
Compound	Conc. (mg/ml)	Control	Test	% decrease	% inhibition (mean % decrease)
Phenergan hydrochloride	2	.21	.00	100	
		2.36	.76	68	
		1.09	.64	41	
		.66	.00	100	77
	4	.87	.10	89	
		.91	.00	100	
		.20	.00	100	
		.52	.00	100	97
Phenergan methosulfate	2	.10	.05	50	
		.04	.02	50	50
	4	.45	.14	69	
		.73	.09	88	79
Pyribenzamine	6	.31	.04	87	
		.13	.00	100	
		.23	.00	100	
		.56	.22	61	87

they employed caused convulsion in one animal and death in another.

The present experiments, begun independently of the foregoing, demonstrate the inhibition of histamine-stimulated gastric acid secretion when considerably lower concentrations of antihistaminics are applied to the mucosa of canine pouches. However, topical application of a surface-active local anesthetic, nupercaine, also inhibits such secretion in comparable degree, and this limits definitive interpretation of these experiments, at least for the time being.

Methods. In this study vagally denervated corpus pouches were used, and the response to histamine (0.1 mg base/kg body weight), given subcutaneously, was measured before and after application of one of several antihistaminics. The inhibitor was instilled into the pouch for a total of 30 minutes, the solution being exchanged for a fresh one at the end of the first 15 minutes. Concentrations of the drug in isotonic sodium chloride ranged from 2 to 10 mg/ml, and volumes of 25-50 ml were required to distend the pouches slightly with hand pressure on the syringe plunger. The antihistaminic compounds used were phenergan, phenergan methosulphate, pyribenzamine, and neoantergan; the local anesthetic agent was nupercaine. In a second series of experiments, gastric secretion was stimulated by the vagomimetic drug, urecholine (carbamyl methylcholine chloride), administered subcutaneously at a dosage of 0.1 mg/kg body weight.

Results. Although the gastric acid secretory response to a fixed dose of histamine may vary considerably from day to day in the same animal, the response to 2 successive doses given to the same animal on the same day (as in the so-called "tandem histamine test") show more regularity. To confirm this, and to control any effect of the application of isotonic saline itself, 10 experiments were performed in which 2 doses of histamine were given at an interval of 2¼ hours, with an application of saline to the pouch for 30 minutes between doses. From Table I, it will be seen that the percentage difference between the first (control) and second (test) responses in output of HCl varied from 0 to 37, with a mean of 10%. In all subsequent experiments using a drug, the doses of histamine were given at the same interval.

The effect of local application of several antihistaminics (phenergan, phenergan methosulphate, pyribenzamine, and neoantergan) on output of histamine-stimulated acid from pouch dogs is also shown in Table I. A significant degree of inhibition of acid output occurred at each dose level with each of these compounds, being greatest with phenergan at a concentration of 4 mg/ml (100% in-

TABLE III. Effect of a Topical Anesthetic (Nupercaine), Applied to Gastric Mucosa of Canine Heidenhain Pouches, on Histamine- and Urecholine-Stimulated Gastric Acid Secretion.

Stimulus	Nupercaine conc. (mg/ml)	Output of HCl (mEq)			% inhibition (mean % decrease)
		Control	Test	% decrease	
Histamine (.1 mg/kg)	1	.79	.82	4	
		2.22	1.53	31	18
	2	2.13	1.40	34	
		3.55	1.34	62	
		2.60	1.35	48	
		3.52	3.07	13	
		.48	.24	50	
		1.34	.87	35	
		2.14	1.62	24	
		.83	.54	35	38
	4	.40	.12	70	
		.75	.17	77	
		.80	.00	100	
		2.25	.98	56	76
Urecholine (.1 mg/kg)	2	.68	.91	0	
		.48	.49	0	
		.61	.57	7	2
	4	.10	.00	100	
		.37	.00	100	100

hibition).

The results of experiments in which urecholine (0.1 mg/kg) was used to stimulate gastric acid secretion are shown in Table II. With this stimulus also, antihistaminic phenergan effected a virtually complete inhibition at a concentration of 4 mg/ml, and significant inhibition was elicited by phenergan methosulphate, and pyribenzamine at concentrations of 4 or 6 mg/ml.

Of considerable interest is the fact that inhibition could likewise be elicited with a representative topical anesthetic. Thus, nupercaine at a concentration of 4 mg/ml induced a mean degree of inhibition of 76% in a number of experiments with histamine stimulation, and 100% in those in which urecholine was the stimulus (Table III).

The topical application of these agents to the pouch mucosa was well tolerated systemically by these animals, even at the low concentrations used. Following instillation of phenergan at 4 mg/ml, there occurred a significant flow of a non-acid mucoid fluid, smaller in volume but similar in appearance to that which has been obtained in a similar investigation in this laboratory, following instillation of the enzyme-inhibitors iodoacetamide and N-ethyl maleimide(3). This happened in both the histamine and the urecholine experiments. None of the other inhibitors gave rise to this mucoid fluid except in several of the experiments with nupercaine in which HCl secretion was prevented entirely. Duration of drainage of this non-acid fluid never lasted longer than 3 hours, and usually only about 2, in contrast with 8 hours or more after the enzyme-inhibitors used in the previous investigation. The specimens were blood-tinged (never frankly bloody) in a few of the experiments, which is believed to have resulted from slight mechanical trauma by manipulation of the catheter during the instillation.

Discussion. The present experiments demonstrate that application of several histamine-blocking agents locally to the gastric mucosa of vagally denervated pouch dogs can inhibit secretory response to histamine in considerable degree. Thus, they confirm the findings of Wood in the cat(*2*), and of Robertson and Grossman in the dog(1). In contrast to the latter authors' report, however, the concentrations of drug used herein were low, no frankly bloody mucus was produced, and no untoward systemic effects of the compounds were elicited. The inhibitory effect of antihistaminic drugs on secretion induced vagomimetically was demonstrated by blocking urecholine-stimulated secretion with several of these agents, thus confirming the report of Robertson and Grossman on pyribenzamine. The finding that a local anesthetic agent can inhibit both histamine-stimulated and urecholine-stimulated acid secretion is also of great interest. In all these experiments, the mean degree of inhibition is clearly related to the dosage employed, and actually reaches 100% in the phenergan-histamine and nupercaine-urecholine experiments.

Robertson and Grossman were reluctant to ascribe any specific significance to the results of their experiments on 2 grounds. First, because of the production of gastric irritation and bloody mucous secretion from the pouch, they ascribed a non-specific role to the antihistaminics employed. Our experiments meet this objection since concentrations of drug used were low, no local irritant effects were produced, and no systemic symptoms were elicited. Some non-acid mucoid fluid was produced with phenergan and nupercaine in those experiments in which acid inhibition was complete. However, the similarity of this material to the mucous secretion obtained after inhibition with N-ethyl maleimide and iodoacetamide, and the fact that the latter is unequivocally differentiated from interstitial fluid or transudate by its content of nitrogen, hexosamine, and reducing substances(3), make it likely that this is some secretory rather than "irritant" inflammatory product. Secondly, inhibition of the response to a parasympathetic stimulant (urecholine) as well as to histamine was interpreted by Robertson and Grossman as evidence definitely against specific antihistaminic action of the drugs. This is not crucial to the argument for specificity, since these same authors have previously shown(4) that hog renal cortex histaminase inhibits urecholine-stimulated gastric secretion, as well as that induced by histamine and food, and it may be argued that a cholinergic mechanism is intermediate in the histamine effect, or vice versa. The recent demonstration in this laboratory(5) of inhibition of histamine acid secretion by atropine is consistent with such an interpretation. On the other hand, the inhibition of histamine-stimulated secretion by the local anesthetic, nupercaine, indicates that there are limitations to interpreting the antihistaminic effect as a specific one, especially since the antihistaminic compounds are also known to be local anesthetics.

Kay and Forrest(6) have also recently reported that the antihistaminic promethazine and mepyramine, when applied locally to the mucosa of Heidenhain pouches, will inhibit the acid secretory response to histamine. They have argued that this is a specific effect rather than a local anesthetic one because, although these two compounds have both local anesthetic and antihistaminic properties, promethazine was more potent than mepyramine in its antihistaminic and its acid inhibitor activity. This argument, however, is of limited force, because these authors did not directly study the effects of local anesthetics, and even more so because the present study indicates that local anesthetics can inhibit histamine- and vagomimetic-stimulated acid secretion when the concentrations of drug are large enough.

Current evidence indicates that there are at least 3 possible modes of action of the antihistaminics in blocking histamine-stimulated acid secretion: (1) a specific antihistaminic effect, (2) the well known atropine effect of these compounds, and (3) their local anesthetic action. In addition to these well-established properties, it is conceivable also that these substances may act by way of some effect on intracellular processes of acid formation. The present experiments do not allow of any definitive choice among these possibilities. Thus, the failure to achieve antihistaminic blocking effects on the stomach by the parenteral administration of antihistaminic compounds is still puzzling, and from the therapeutic point of view an extremely disappointing phenomenon.

Summary and conclusions. 1. Topical application of several antihistaminic drugs to the mucosa of canine corpus pouches significantly inhibited the acid secretory responses of these pouches to parenterally administered histamine. One local anesthetic agent elicited similar inhibition. These inhibitory substances were effective also against a vagomimetic stimulant, urecholine. 2. In an effort to explain these phenomena, 3 possible modes of action of the antihistaminics present themselves: (1) a specific (antihistaminic) effect, (2) an atropine effect, (3) a local anesthetic effect. The evidence presently available does not as yet permit of any preference among these several hypotheses.

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Efficiency of Intestinal Absorption of Vitamin B_{12} Measured by Hepatic Uptake of $Co^{60}B_{12}$.* (23208)

GEORGE B. JERZY GLASS AND ROGER W. LAUGHTON
Department of Medicine and Gastroenterology Research Laboratory, N. Y. Medical College, Flower & Fifth Avenue Hospitals, N. Y. City.

In an earlier work(1) we studied the intestinal absorption of vit. B_{12} by means of measurement of hepatic uptake of $Co^{60}B_{12}$ (2). Booth and Mollin had compared this method with measurement of fecal excretion of $Co^{60}B_{12}$ and showed that hepatic uptake of radioactive B_{12} a week after oral administration is proportional to amount of B_{12} absorbed in the intestine(3). We have also expressed the efficiency of intestinal absorption of vit. B_{12} in terms of parenteral equivalents (1), by repeating measurement of hepatic uptake in the same individual after oral and parenteral administration of similar doses of $Co^{60}B_{12}$. Pollycove and Apt(4) demonstrated that the determination of efficiency of intestinal absorption of B_{12} by this method gives results similar to those obtained by measurement of fecal excretion of $Co^{60}B_{12}$. The present investigation was undertaken to add more information on the efficiency of intestinal absorption of vit. B_{12} under various pathological conditions, by this technic.

Method. Hepatic uptake of $Co^{60}B_{12}$ was measured by a previously described method (2) on 31 patients with a variety of diseases, and a total of 112 individual tests were made. The determinations were done 6-7 days following a) oral administration of tracer dose of 0.5 μg $Co^{60}B_{12}$ containing 0.35 resp. 0.50 μc Co^{60}†, b) same dose of $Co^{60}B_{12}$ together with potent intrinsic factor preparation‡, c) same dose of $Co^{60}B_{12}$ intramuscularly. Each of these 3 tests was done on the same patient at intervals of 10-14 days. This is the time interval at which a plateau of hepatic counts appears(2-6). This leveling off of radioactivity over the liver makes it possible to use final radioactivity counts in one test as a base line for the next. As many as 4-6 individual tests were done on 12 patients. Efficiency of intestinal absorption of B_{12} was calculated in terms of parenteral equivalents according to the formula:

$$\text{Parenteral equivalent of intestinal absorption (in \%)} = \frac{\text{Hepatic uptake on oral admin.}}{\text{Hepatic uptake on parenteral admin.}} \times 100.$$

The cases included 7 patients with pernicious anemia, 4 with sprue, 3 with macrocytic

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† Supplied through courtesy of Drs. N. Ritter and Ch. Rosenblum of Merck Co., Inc., Rahway, N. J.

‡ Supplied through the courtesy of Dr. K. W. Thompson of Organon, Inc., Orange, N. J. The preparation used (#5333) did not contain any appreciable amount of B_{12} on microbiological assay and was used at dose of 62 mg, equivalent to dose contained in 2 USP units of oral anti-anemia preparation.

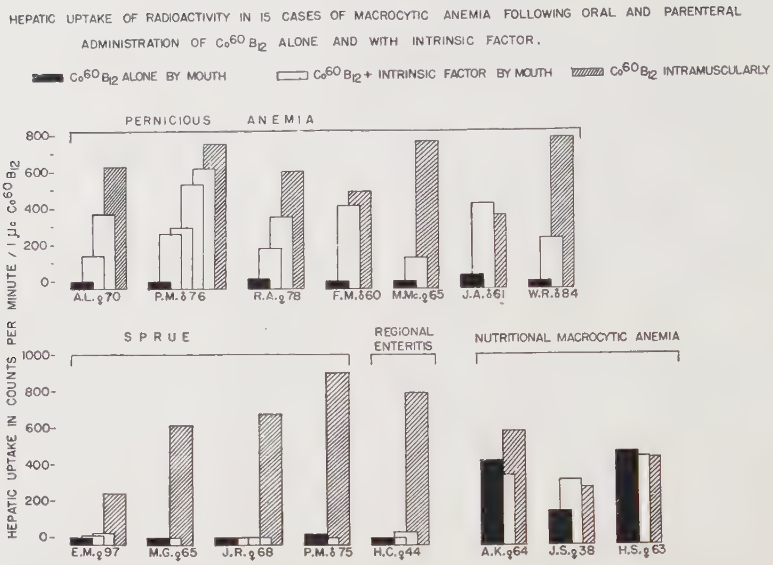

FIG. 1. Hepatic uptake of radioactivity in 15 cases of macrocytic anemia following oral and parenteral administration of $Co^{60}B_{12}$ alone and with intrinsic factor.

non-pernicious anemia, 3 patients following total, and 3 following subtotal gastrectomy, 3 with hepatitis, 2 patients with histamine-fast anacidity, 2 with cirrhosis of the liver, 1 with regional enteritis, and 3 normal controls. The hepatic uptake in 3 cases of total and 1 case of subtotal gastrectomy was counted at a higher voltage with somewhat different equipment. This accounts for relatively higher counts than that obtained in the remaining cases.

Results. These are shown in Fig. 1 and 2. The efficiency of intestinal absorption of $Co^{60}B_{12}$ in various patients, as calculated in parenteral equivalents of absorption, ranged from 0 to 113%. No absorption was noted in 5 out of 7 cases of pernicious anemia, in 3 out of 4 cases of sprue, in the 3 cases of total gastrectomy, and in one case of regional enteritis. It was only 3% and 10% each in the 2 remaining cases of pernicious anemia in remission, and 2% in the remaining case of sprue. No hepatic uptake was noted in a case of hepatic cirrhosis with hepatic insufficiency, studied a few days before death, whereas it had been normal 2 weeks before. In the other cirrhosis case, in one case of hepatitis, and in one case of histamine-fast anacidity without other symptoms, the efficiency of intestinal absorption was 24-38%, *i.e.* below the lower limits of normal, which is about 40% at a dose of 0.5 μg B_{12} orally administered(6). Efficiency of B_{12} absorption was within normal range (40-109%) in the 3 cases of subtotal gastrectomy, in the 3 cases of macrocytic non-pernicious nutritional and hemolytic anemia, in 2 of the 3 cases of hepatitis, in one of the 2 cases of histamine-fast anacidity, and in the 3 control cases.

Addition of intrinsic factor preparation markedly raised efficiency of intestinal absorption of B_{12} in all 7 instances of pernicious anemia (from 0-14% to 16-113%), and also raised it in the 3 cases of subtotal gastrectomy, in one of the 3 cases of histamine-fast anacidity, in one of 3 cases of cirrhosis, and in one case of the macrocytic non-pernicious anemia (Fig. 1 and 2). The intrinsic factor had no effect upon B_{12} absorption in the 3 cases of sprue, and the effect was only very slight in the case of the one other sprue patient. Only in one case of hepatitis and in one control, did we note a marked inhibitory effect by the intrinsic factor preparation used upon efficiency of intestinal B_{12} absorption. In these 2 cases B_{12} absorption decreased from 63-80% and 47% respectively to 9% in both instances.

Comment. It is not known whether the same percentage of B_{12} is deposited in the

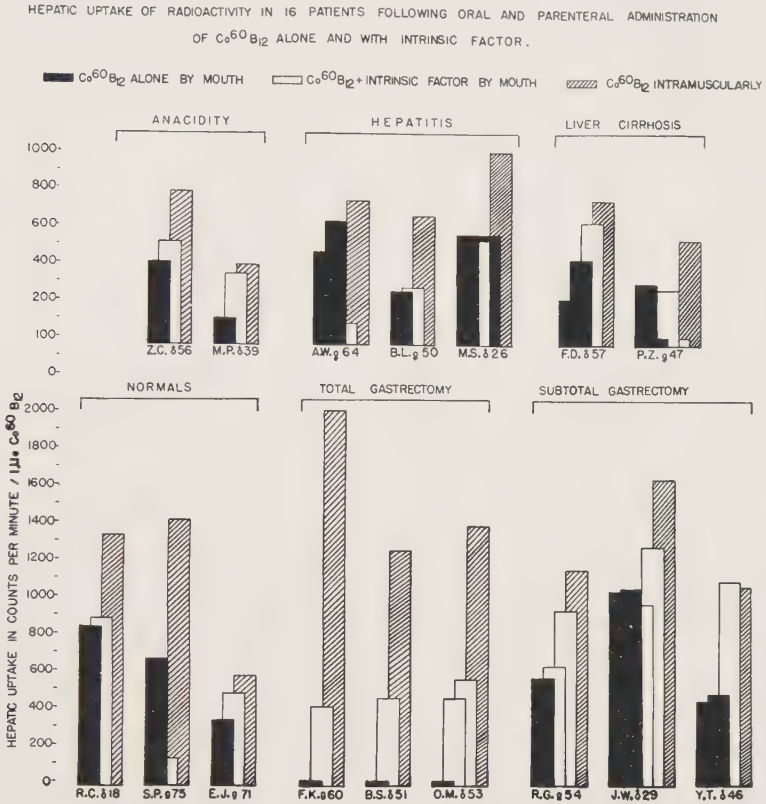

FIG. 2. Hepatic uptake of radioactivity in 16 patients following oral and parenteral administration of $Co^{60}B_{12}$ alone and with intrinsic factor.

liver when given intramuscularly, as when absorbed upon oral administration. The method used in this study, therefore, does not quantitate the intestinal absorption of vit. B_{12}, but provides a way to express the efficiency of absorption of B_{12} in terms of parenteral equivalents of intestinal absorption. It supplies comparable values which agree very well(4) with the figures derived from the method of measuring fecal excretion of Co^{60} B_{12}. This agreement is due to the fact that a) the hepatic deposition of B_{12} at the end of the first week is proportional to the amount of B_{12} absorbed in the intestine(3) and to the dose injected intramuscularly(7-8); b) if the i.m. administered dose of vit. B_{12} is small, as was the case in these studies, B_{12} is completely absorbed within a few hours at the site of injection(7-8) and will be excreted in the urine only in traces(9); c) the pattern of hepatic deposition of vit. B_{12} after oral (2-5) and parenteral administration(7-8) is similar and shows a slow rise, which comes to a peak at the end of the first week. It levels off thereafter. Proportionately, more B_{12} is deposited, however, in the kidneys after parenteral injection(15) than is the case after oral intake(10). Following oral administration, therefore, the distribution of B_{12} in the body favors the liver more than when B_{12} is injected, which would cause higher values of parenteral equivalents of intestinal absorption. This, in turn, would indicate somewhat higher efficiency of intestinal absorption than is really the case.

Our actual data on the abolished intestinal absorption of vit. B_{12} in pernicious anemia, sprue, and after total gastrectomy, correction of this defect by intrinsic factor preparation, and the lack of effect of intrinsic factor upon B_{12} absorption in sprue, are in line with our previous observations obtained by measurement of hepatic uptake of $Co^{60}B_{12}$ after its oral administration only(2,11-13). Efficiency of intestinal absorption of B_{12}, calculated in terms of parenteral equivalents, is more suitable for comparison than figures of hepatic uptake of B_{12} obtained in various individuals. The shape, size and position of the liver and the crystal of the scintillation counter influence the hepatic count. Calculation of efficiency of intestinal absorption of B_{12} in terms of parenteral equivalents disposes of these technically interfering elements. For clinical purposes, however, such as differentiation of macrocytic anemias, or detection of asymptomatic forms of pernicious anemia or sprue, calculation of parenteral equivalents of intestinal absorption has no advantages. Here the simplified, 48-hour technic of measuring hepatic uptake of radioactivity following oral administration of $Co^{60}B_{12}$, alone or with intrinsic factor, is entirely adequate and satisfactory(14).

Summary and conclusions. Hepatic uptake of radioactivity following oral administration of a tracer dose of $Co^{60}B_{12}$ alone and with intrinsic factor preparation was compared with that after intramuscular injection of the same dose of $Co^{60}B_{12}$ on 31 individuals with various diseases. Efficiency of intestinal absorption and effect of intrinsic factor preparation was quantitated in this way and was expressed in terms of parenteral equivalents of intestinal absorption.

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Effect of *H. pertussis* on Sensitivity of Mice to Serotonin. (23209)

J. MUNOZ (Introduced by W. F. Verwey)
(With technical assistance of M. A. Greenwald)
Merck Institute for Therapeutic Research, Rahway, N. J.

It is well known that injection of *Hemophilus pertussis* cells to mice of certain strains produces a marked increase in histamine sensitivity(1,2,3) as well as an increase in ability of mice to become anaphylactically sensitive (4,5). Since histamine plays an important role in anaphylaxis of guinea pigs and other animals as well as man(6), it was tempting to conclude that pertussis increased susceptibility of mice to anaphylaxis mainly by increasing their susceptibility to histamine(7). This, however, does not explain why some strains do not become sensitive to histamine after pertussis injection(3,11) while becoming highly susceptible to anaphylaxis(11). Moreover, the state of histamine hypersensitivity in susceptible strains disappears much sooner than that of anaphylactic sensitivity.

TABLE I. Effect of *H. pertussis* Injection on Toxicity of Serotonin to Mice.

	Results							
	TF mice				CF mice			
Serotonin, mg/kg	Normal		Pertussis treated		Normal		Pertussis treated	
	D/T*	% D	D/T	% D	D/T	% D	D/T	% D
1.17	0/15	0	0/15	0	0/15	0	0/15	0
2.34	"	0	9/35	25.3	"	0	3/14	21.2
4.68	"	0	24/33	72.7	"	0	6/15	40.0
9.37	"	0	24/34	70.5	"	0	10/15	66.6
18.75	"	0	20/33	60.6	"	0	9/14	64.2
37.50	"	0	22/33	66.7	2/15	13.3	12/14	85.6
75.00	1/15	6.6	18/35	51.4	0/15	0	13/15	86.6
150.00	13/15	86.7	11/15	73.4	9/15	60	10/15	66.6

* D/T = Deaths/Total mice inj.

For these reasons, it appears that in anaphylaxis of pertussis treated mice, hypersensitivity to circulating histamine does not play an important part. Other mechanisms must then be sought. The role that serotonin (5-hydroxytryptamine) may play in anaphylaxis has been suggested by various investigators. In guinea pigs serotonin can produce symptoms that are similar to those observed in anaphylaxis(8), and it is released *in vitro*, together with histamine, from platelets by an antigen-antibody reaction(9). Fink(10) found that serotonin inhibitors [d-lysergic acid diethylamide (LSD) and reserpine] prevent the Shultz-Dale reaction. For these reasons, it was of interest to investigate the effects of *H. pertussis* on sensitivity of mice to serotonin.

Materials and methods. Female mice weighing from 14-16 g of either CF-1 strain (from Carworth Farms) or TF strain (Swiss Webster mice from Tumblebrook Farms) were used. Mice were injected intraperitoneally with 2,000 million killed smooth *H. pertussis* cells suspended in 0.25 ml of physiological saline solution. Mice were challenged intravenously with various doses of serotonin* 4 days after pertussis injection. The drug was dissolved in physiological saline solution. Although final results were recorded 24 hours after injection of serotonin, most deaths occurred within the first 2 hours.

Results. Effect of H. pertussis on susceptibility of mice to serotonin. Groups of TF and CF-1 mice injected with pertussis cells were challenged with various doses of serotonin. The two strains were used because it is known that the TF mice become highly sensitive to histamine after injection of pertussis, while CF-1 mice do not(3). This latter strain should not be confused with the CF-W strain that is available from the same source. The results obtained are summarized in Table I. It is clear from these results that both TF and CF-1 mice become 20-30 times more sensitive to serotonin after administration of *H. pertussis*. There is an indication that TF mice may become slightly more sensitive than CF-1, but the difference is not significant. With TF mice there is also a suggestion that the dose response curve for serotonin is a biphasic type of curve similar to that found previously with histamine(12). At 4.68 mg/kg dose of serotonin 72.7% of the animals died, while at 75 mg/kg dose only 51.4% died.

Optimal time for development of serotonin sensitivity. Groups of TF mice were given pertussis cells, and at various intervals of time thereafter were challenged with various doses of serotonin. The results obtained are given in Table II where it can be seen that a low degree of sensitivity to serotonin was ob-

TABLE II. Development of Sensitivity to Various Doses of Serotonin after *H. pertussis* injection.

Serotonin, mg/kg	Days after *H. pertussis* 1	3	6	8	10
2.34	0/10*	4/10	4/ 9	2/10	2/10
4.68	1/10	9/10	6/10	5/10	1/10
9.37	4/10	5/10	5/10	5/10	8/10
18.75	3/10	9/10	5/10	6/10	4/ 9
37.50	3/10	4/10	7/10	3/10	8/10

* Deaths/Total mice inj.

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* Purchased as creatinine sulfate salt from Nutritional Biochemicals Corp., Cleveland, O.

TABLE III. Development and Disappearance of Serotonin Sensitivity in *H. pertussis* Mice.

Group	Days after *H. pertussis*	Results, D/T
1	—*	0/20
2	1	0/20
3	2	7/20
4	4	15/20
5	11	12/20
6	18	8/20
7	25	2/20
8	32	1/20
9	—*	1/10
10	—*	0/ 9

* No pertussis. A control group of 20 mice from the same batch of animals was challenged on the same day as Group 2, and another group of 10 normal mice on the same day as Group 7 and the last control group on the same day as Group 8.

TABLE IV. Effect of Various Antigens on Development of Sensitivity to Serotonin.

	Results			
Serotonin, mg/kg	*H. pertussis*	*S. typhosa*	*E. rhusiopathiae*	Normal
2.34	2/ 9*	0/10	0/10	0/10
4.68	7/10	”	”	”
9.37	”	”	”	”
18.75	5/ 9	”	”	”
37.50	5/10	”	”	1/10
75.00	6/10	”	”	0/10

* Deaths/Total inj.

served one day after pertussis injection. By the third day optimal sensitivity was obtained and it persisted for 10 days when the experiment was terminated. These results show that sensitization of mice to serotonin varies among individual mice just as in the case of histamine sensitization(3).

To establish how long sensitivity to serotonin lasts, another experiment was performed extending the period of observation to 32 days. One-hundred-eight TF mice were used, of which 140 received *H. pertussis* cells and 40 were kept as controls. Groups of 20 mice each were challenged at various intervals after administration of *H. pertussis* with 18 mg of serotonin per kg of body weight. From the results given in Table III, as well as those in Table II, it is clear that sensitivity to serotonin reaches its peak three to four days after *H. pertussis* injection and gradually decreases until sensitivity returns to normal by the 25th day. Groups of normal control mice challenged at the beginning and at the end of this experiment (groups 1, 9 and 10, Table III) showed no marked sensitivity to 18 mg of serotonin per kg of body weight.

Effect of other bacterial cells on sensitivity of mice to serotonin. Three different bacterial suspensions were used—acetone-dryed *Salmonella typhosa* cells, *Erysipelothrix rhusiopathiae* and *H. pertussis*. One group of mice received intraperitoneally 0.25 ml of a suspension of *S. typhosa* cells containing 0.032 mg N/ml, another group received 0.25 ml of suspension of *E. rhusiopathiae* of a turbidity equivalent to 8,000 million *H. pertussis* cells/ml, and the last group received 0.25 ml of suspension of *H. pertussis* containing 8,000 million cells/ml. Four days later the mice were challenged with serotonin. The results are given in Table III. Of the 3 bacterial species used, only *H. pertussis* was capable of producing the sensitivity to serotonin.

Discussion. It has been difficult to account for the increased susceptibility to anaphylaxis induced in mice by *H. pertussis*. Although in some strains of mice the increase in susceptibility to histamine was thought to explain the greater susceptibility to anaphylaxis(7), other strains failed to sensitize to histamine(3,11) but became more susceptible to anaphylaxis after pertussis treatment (11). This observation suggested that other mechanisms, besides histamine action, may be involved in mouse anaphylaxis. Although, from the results obtained in our experiments, as well as those reported by others(8,9,10), it is tempting to suggest that pertussis cells exert their effect by increasing the sensitivity of mice to serotonin, no direct proof to support this view has been obtained. Fink's observations that LSD and reserpine inhibit specific contraction of mouse uterus *in vitro,* tend to support the serotonin hypothesis. It should be remembered, however, that both LSD and reserpine are not specific anti-serotonin drugs, and it may well be that inhibition observed by Fink was due to a property other than that of inhibiting serotonin. Although the anti-serotonin activity of LSD as well as other anti-serotonin compounds like BAS can be shown by protecting pertussis treated mice against lethal doses of serotonin, it has not been possible with these substances to protect either mice or guinea pigs from anaphylaxis. In fact, BAS actually seems to enhance anaphylactic deaths.† For these reasons, it is premature to assume that pertussis cells exert their anaphylactic-enhancing effect by increasing the sensitivity to serotonin. It should be noted, however, that the type of death observed in pertussis-treated mice after injection of lethal doses of serotonin or histamine is grossly indistinguishable from that observed in anaphylaxis(13). Typically, after injection of serotonin mice become lethargic. Respiration becomes forced and within a few minutes signs of cyanosis are observed as evidenced by the color of snout, tail, ears and paws. The snout actually appears to be swollen. Little cyanosis is observed in non-lethal reactions. Mice usually die within 15-30 minutes giving few tonic contractions of the hind legs. Usually after these final convulsions mice continue to gasp for a short time. The similarity of death produced by these three different agents may well point to a common mechanism, and as Herxheimer(8) has suggested, death may result from a still unknown mechanism triggered by antigen-antibody reactions as well as by serotonin, histamine and perhaps other substances.

Summary. (1) Two strains of mice known to differ with respect to their ability to become sensitized to histamine, were found to become highly sensitive to serotonin after the injection of *H. pertussis* cells. (2) Some sensitivity to serotonin could be detected 16-24 hours after the administration of pertussis. Sensitivity reached its peak by the third to fourth day, and disappeared by the 25th day after pertussis injection. (3) Ability to induce sensitivity to serotonin seems to be characteristic of *H. pertussis*.

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† Unpublished observations.

Cytotoxic Effect of Nephrotoxic Serum on Rat Tissue Culture.* (23210)

CHARLOTTE T. LIU, WALLACE W. MCCRORY AND JOHN A. FLICK
(Introduced by Werner Henle)
Children's Hospital of Philadelphia, Department of Pediatrics, School of Medicine, Department of Microbiology, Graduate School of Medicine, University of Pennsylvania.

Lippman(1) reported that gamma globulin made from rabbit anti-rat-kidney serum inhibited growth in tissue culture explants of rat heart muscle and kidney as well as chick-embryo-brain, heart muscle and mesonephrons. The cytotoxicity of this serum did not appear species specific. Investigation of this problem has been renewed in our laboratory using monolayer tissue culture cells(2). This report concerns the effects of anti-rat-

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kidney serum on (1) rat kidney cells *in vivo* (nephrotoxicity), (2) rat kidney cells in tissue culture, (3) hemagglutination test and (4) the interrelationships.

Methods. Preparation of tissue culture. Kidneys of rats or other species were minced and trypsinized according to Youngner's technic(2). The renal cells were suspended in a medium containing 10% inactivated calf serum and 0.5% lactalbumin in Hanks balanced salt solution. One ml aliquots of this cell suspension were delivered into tissue culture tubes. After 3-4 days of incubation, the tubes were refed. On the same or the next day of refeeding, varying concentrations of antisera were added to the medium to give final dilutions of the serum from 1:6 to 1:192 in 2-fold steps. *Production of immune sera.* New Zealand rabbits weighing 2-3 kg each were used in pairs for producing various antisera against: 1. Saline organ emulsions: Perfused rat kidney, liver, lung and heart were macerated and prepared as 20% suspensions in physiological saline. They were given intraperitoneally by a schedule similar to Ehrich, Forman and Seifter(3). 2. Tryptic digest of rat kidney: This material was prepared by the method described by Cole(4). The material thus prepared had a total nitrogen content of 263 mg%. A total amount equivalent to 7-9 g of the original organ was given to each animal intravenously over a period of 8-10 weeks. A more concentrated tryptic digest of kidney was made with 500 mg% of nitrogen. This was injected intraperitoneally once a week over a period of 7 weeks with a total dosage equivalent to 21 g of original kidney tissue. 3. Glynn's antigen of kidney digest adsorbed onto streptococci: Streptococci of group A type 12 and concentrated rat tryptic kidney digest was used in the preparation as described by Glynn and Holborow(5). 4. Human kidneys: Normal infant kidneys obtained shortly after death were emulsified in a Waring Blendor. The 20% kidney emulsion was injected into rabbits intraperitoneally. Human kidney obtained at surgery was immediately planted into tissue culture bottles(2). After several passages, the sheets of cells were washed and collected. Five ml of a 5% cell suspension in saline was injected 3 times intraperitoneally and 6 times intravenously into rabbits in 6 weeks. All the above animals were bled from the heart 4-10 days after the last injection. The antisera were inactivated at 56°C for 30 min., adsorbed once with washed rat blood cells and then stored at −20°C until used. *Definition of cytotoxicity.* After antisera were added, renal tissue cultures were observed for cellular damage by low power microscopy daily for a period of 5 days. Cytologic changes were as follows: Mild or early effects were apparent as clumping of cells, nuclei became pyknotic and the cytoplasm became "dusty" and coarsely granular. There was shrinking of the cytoplasm of these cells sometimes leaving thready spicules extending from one cell to another. Dead and pyknotic cells appeared first along the margins of the sheets. This process continued until all cells disappeared from the walls of the culture tubes. We arbitrarily defined unequivocal cytotoxicity as the loss of at least 50% of the cells (as compared to control culture tubes) and alterations of the type detailed above in the remaining cells. *Method of absorption.* To each 2 ml of nephrotoxic sera, 0.75 ml of organ material was added separately. The materials used were digests from 0.4 g wet weight of rat lung, liver, heart and kidney and 50% whole kidney emulsion. The nephrotoxic serum was adsorbed with living rat kidney tissue culture cells by replacing the culture medium with 1 ml of nephrotoxic serum and then incubating at 37°C overnight. This was repeated 6 times. *Hemagglutination test.* Boyden's method(6) was adopted with slight modification in that 1:10,000 tannic acid and 5% sheep red cells were used. The tryptic digest antigen used for adsorption onto the tanned sheep blood cells contained approximately 2.5 mg N/ml. *Bioassay of nephrotoxic sera.* Long-Evans rats, weighing 100-150 g each, were used. For the rabbit anti-rat-kidney sera, 0.1-1 ml of serum was given intravenously per rat in a single or 2 divided doses. The other organ antisera were also given intravenously as 0.5-3 ml per rat in a single or 2-3 divided doses on 2-3 consecutive days. Eighteen hour urine specimens were collected on 1, 3, 7, 10 and 14 days

TABLE I. Comparison of Anti-Kidney Antibody Titers, Nephrotoxicity and Cytotoxicity of Various Antisera.

Rat tissue used for immunizing rabbit	Hemagglutination titers *vs*				Nephrotoxicity in rats	Cytotoxicity for rat renal tissue culture
	Kidney	Liver	Lung	Heart		
Kidney emulsion	40,960	<40	<40	640	++++	96*
" digest, I.V.	640	"	"	160	0	6
I.P.	160	"	"	<40	0	<6
Kidney digest adsorbed onto strep.	2,560	"	"	"	+++	24
Lung emulsion	640	"	40,960	640	0	6
Liver "	<40	40,960	2,560	"	0	<6
Heart "	"	160	<40	"	0	"
Streptococci	"	<40	"	<40	0	"
Normal rabbit sera and zero time	"	"	"	"	0	"

* Reciprocal of highest significant serum dilution.

after the last injection. The rats which survived until the end of the 2 weeks of observation were sacrificed. Histological examination of all kidneys was made by Dr. Bernard Wagner, associate pathologist of the hospital.

Results. From Table I, it is evident that the tryptic digest of rat kidney was only weakly antigenic. By adsorbing the soluble kidney digest onto streptococci, the kidney digest was apparently converted into a better antigen. The most potent nephrotoxic sera *in vivo* were those produced by injecting whole kidney emulsion. This was true in all parameters. Their anti-kidney hemagglutinin titers were highest, and they showed cytotoxic effects at 1:96 dilution of the sera in rat renal tissue culture.

The organ specificity of this cytotoxic effect is indicated by the results of similar tests with the other rat organ antisera. Only the anti-lung serum demonstrated slight cytotoxic effects. Normal rabbit sera (including inactivated sera of those rabbits used for producing various antisera obtained prior to period of immunization) were not cytotoxic.

Organ specificity was assayed further by comparing the adsorbing ability of standard doses of tryptic digests of various organs (Table II). While the amount of kidney digest used in this adsorption was insufficient to completely neutralize the nephrotoxicity and cytotoxicity of the nephrotoxic serum it did neutralize the antiserum made by injecting rat kidney digest adsorbed on streptococci. Lung digest was much more effective than digests of liver and heart in lessening reactivities in all parameters. The nephrotoxic serum, after adsorption with living rat kidney culture cells, was no longer cytotoxic in tissue cultures or nephrotoxic when injected in dose of 0.5-1 ml into rats, and the anti-kidney hemagglutinin titer was greatly reduced, (1:160).

Species specificity was demonstrated by using: (1) the usual nephrotoxic serum (for rats), (2) rabbit antiserum prepared against human kidney cells grown in culture and (3) rabbit antiserum prepared against whole human kidney emulsion. These sera in various concentrations were tested for cytotoxic effects in cultures of kidney cells derived from 6 species of animals. The nephrotoxic serum appeared most toxic for Long-Evans rat kidney cells and slightly toxic for white rat and DBA mouse kidney cells (Table III). However, it was nontoxic for other kidney cells. The antiserum against human kidney tissue culture cells was cytotoxic for human cells and weakly cytotoxic for monkey kidney cells. The antiserum against human kidney obtained from autopsy had toxic effects only for human kidney tissue culture cells.

Discussion. A correlation has been demonstrated to exist between anti-kidney hemagglutinin titers and *in vivo* nephrotoxicity in

TABLE II. Assay of Organ Specificity by Absorption Test.

Serum and added component	Anti-kidney titer (HA)	Nephrotoxicity in rats	Cytotoxicity in tissue culture
1. Anti-rat-kidney serum			
+ physiological saline	1:40,960	++++	1:96*
+ rat kidney emulsion	<1: 40	0	<1: 6
+ " " digest	"	+	1: 6
+ " lung "	1: 640	++	1:24
+ " liver "	1: 1,280	++++	1:48
+ " heart "	1: 2,560	++++	1:48
+ " kidney tissue culture cells	1: 160	0	<1: 6
2. Anti-strep.-adsorbed-kidney-digest serum			
+ physiological saline	1:2,560	+++	1:24
+ rat kidney emulsion	<1: 40	0	<1: 6
+ " " digest	"	0	"

* Dilution of sera demonstrating unequivocal cytotoxicity for rat kidney tissue culture cells.

these studies. Those sera demonstrating maximal nephrotoxicity had the highest titers of hemagglutinins. Sera lacking nephrotoxic effects in general had hemagglutinin titers of kidney antigens of less than 1:640. These results are in agreement with the recent report of Rothenberg, *et al.*(7). The range of hemagglutination titers also correlated well with the *in vitro* cytotoxic effects. Absorption of the nephrotoxic sera by digests of lung, heart and liver revealed a disproportional reduction in hemagglutinin titers in comparison to the neutralizing effect on nephrotoxicity and cytotoxicity. This would seem to indicate that the antigen-antibody systems measured by the hemagglutination system may differ in some respects from those responsible for nephrotoxicity and cytotoxicity.

We have also employed Middlebrook's(8) method of adsorbing the soluble tryptic digests of organs on sheep red cells not treated with tannic acid. Results with such cells in hemagglutination test were consistently negative. This suggests that the antigen-antibody system being measured by this test does not involve polysaccharide antigens of the kidney.

Baxter and Goodman(9) found that the nephrotoxic antibodies could be removed by adsorption of nephrotoxic sera with extracts of rat organs other than kidney though the quantity needed varied with the different organs. The results of the comparison of the neutralizing potency of the various organ preparations we used for adsorption of nephrotoxic sera are in agreement with their finding. Though these results can be interpreted as indicating some degree of organ specificity, they also could reflect differences in the quantitative distribution of a common antigen in the various tissues.

The data presented suggested that the one or more antibodies causing nephrotoxicity in the intact animals may be identical with the one or more antibodies causing cytotoxicity in culture. The evidence for this is based on the correlation between the cytotoxicity and the nephrotoxicity of the sera and the elimination of these effects by absorption of the sera with

TABLE III. Species Specificity of Cytotoxic Effect.

Rabbit antiserum against:	Source of kidney tissue culture cells Long-Evans rat	White rat	DBA mouse	Rabbit	Monkey	Human
Rat kidney emulsion	1:96	1:24	1:12	<1:6	<1:6	<1: 6
Human kidney tissue culture	<1: 6	<1: 6	<1: 6	"	1:6	1:48
Human (autopsy) kidney emulsion	"	"	"	"	<1:6	1:24
Rabbit serum control*	"	"	"	"	"	<1: 6

* Sera obtained from rabbits prior to production of various antisera.

either whole kidney emulsion or kidney tissue culture cells. The cytotoxic effect in tissue culture of nephrotoxic sera appears to be species specific, though there is some cross reaction between closely related species of animals. This is significant in view of the species specificity of nephrotoxic serum nephritis (9).

Latta and Kutsakis(10) recently reported that a heat-labile, complement-like factor present in guinea pig anti-chick-embryo-heart serum was necessary for the immediate cytotoxic effect on chick-embryo-heart fibroblast grown in tissue culture. We are currently studying the effect of complement-like factors in this cytotoxic system.

Summary. Rabbit antisera to both rat kidney saline homogenate and the tryptic digest of rat kidney adsorbed on streptococci caused cytotoxic effects upon rat kidney cells in tissue culture as well as renal pathology *in vivo* following intravenous injection of rats. The titers of the cytotoxic effect in tissue culture paralleled the nephrotoxic effect *in vivo* and the anti-kidney hemagglutinin titers *in vitro*. The kidney cells grown in tissue culture tubes were effective in absorbing the potent nephrotoxic factor from serum. The cytotoxic effect of the nephrotoxic serum appears to be markedly species specific.

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A Plate Technic for the Conglutinative Complement Fixation Test. (23211)

OTTO G. BIER, RAUL FURTADO[*] AND EDUARDO CISALPINO[†] (Introduced by Z. Ovary)
Department of Microbiology, Escola Paulista de Medicina, São Paulo, Brazil.

The term conglutination (*L. conglutinatio*, act of glueing together) was applied by Bordet and Streng(1) to describe clumping of red blood cells sensitized with antibody in the presence of complement (C′) and of a substance called conglutinin, existing in serum of normal bovines. Realizing that C′ was necessary for conglutination, Streng(2) developed a new serological test applied to the diagnosis of numerous diseases such as syphilis, glanders, dourine, brucellosis, etc. Interest in this test was revived recently largely as a result of the investigations of Hole and Coombs(3-6) and of Rice *et al.*(7,8). Hole and Coombs(3) suggested the expression "Conglutinating Complement Absorption" for Streng's serodiagnostic test to indicate the analogy to and difference from the hemolytic complement fixation (C′F) test. However, use of the word absorption instead of fixation does not seem entirely justifiable, since the same basic phenomenon, *i.e.* disappearance of C′, is involved in both cases. We shall refer, therefore, to "Conglutinative Complement Fixation" (CC′F). Recent applications of the conglutination C′F test have been made in serodiagnosis of rickettsial and viral infections(9-12), such as foot-and-mouth disease (Solovieff,9), Q fever (Wolfe and Kornfeld,10), psittacosis-lymphogranuloma, vaccinia and influenza (Stoker, Coombs and Bedson,12); (Hilleman, Haig & Helmold, 11). Experiments of this kind are frequently handicapped by the small amounts of antigen or antiserum available and this led Fulton and Dumbell(13) to devise a drop method for performing the hemolytic C′F test. This technic has been successfully applied to C′ fixation studies with influenza(13), Coxsackie(14,15) and poliomyelitis viruses (16).

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[*] Fellow of Rockefeller Foundation, on leave of absence from Dept. of Clinical Medicine, Univ. of Santiago Med. School, Chile.

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In the present paper a drop method is also described for the conglutinative C′F test. The conglutinative indicator permits clearer readings than those afforded by the hemolytic test. Furthermore, preparation of glass dropping pipettes, as well as elaborate racks and air-tight boxes required for Fulton and Dumbell's technic are not necessary for the conglutinative C′F microtechnic herein described.

Materials and methods. Six samples of rabbit anti-bovine serum albumin, 21, 22, 24, 25, M and O, containing respectively 0.707, 0.510, 1.080, 0.169, 0.151 and 0.350 mg antibody N/ml were used. The sera were diluted so as to contain 40 μg AbN/ml and preserved frozen at –20°C. Hyperimmunized guinea pig sera were utilized in experiments with foot-and-mouth disease virus, types A and C. The rabbit anti-C-reactive protein (anti-CRP) was a commercial preparation from Schieffelin & Co., New York City. Crystallized bovine serum albumin obtained from Pentex Inc., Kankakee, Ill., was used without further purification. The foot-and-mouth disease antigens were prepared from guinea pig membranes.‡ Beef serum obtained from recent bleeding, and inactivated for 30 minutes at 56°C was used as source of *conglutinin.* The *conglutinative complement* used was fresh, unheated horse serum. Both *complement* and conglutinin were stored frozen in 1 ml amounts at –20°C. The *diluent* used for all reagents was isotonic veronal bicarbonate buffer, pH 7.3-7.4, containing 0.144 M NaCl, 0.00015 M $CaCl_2$ and 0.0005 M $MgCl_2$(17). The *indicator system* was prepared by mixing 2 volumes of sheep red cell suspension containing approximately 1.5 x 10^8 cells/ml with 1 volume of appropriate dilution of rabbit amboceptor and 1 volume of selected dilution of conglutinin. The red cell suspension was first standardized so that an optical density of 0.56 was obtained when 1 ml of the suspension was lysed with 14 ml of distilled water. The O.D. = 0.56 refers to the reading in a Coleman Junior spectrophotometer, model 6, at a wave length of 550 mμ and with matching square cuvettes, 13 x 13 x 100 mm. From this standardized suspension a 1:6.6 dilution was prepared to yield approximately 1.5 x 10^8 erythrocytes/ml. The amboceptor dilution corresponded to the optimal sensitizing dose as usually determined in hemolytic tests with a 10^9/ml red cell suspension. The test-dose of conglutinin was ascertained in bloc titration experiments as indicated in the experimental part. Most tests were performed simultaneously by the usual tube method and by the drop technic. In the latter instance, the container was a plastic polysterene molded plate of the type commonly utilized for cellular cultures and virus neutralization studies.§ During the test, the molded chambers of the plastic panel were partially immersed in ice water, a precaution which was essential to avoid C′ deterioration. After distribution of the reagents and careful mixing of the contents, the panel was covered with a glass plate whose edges were sealed with a strip of Scotch tape. The molded panel was then placed in a metal box whose bottom was covered with a layer of moist absorbent cotton. The unit volume of antiserum, antigen, C′, conglutinin and sensitized cells was 0.2 ml for tests in tubes and 0.05 ml for the plate test, giving final

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‡ The authors are indebted to Dr. Maria Siqueira, Instituto Biologico, São Paulo, Brazil, for supplying foot-and-mouth disease antigens and antisera, types A and C.

§ May be purchased from Dow Chemical, Midland, Mich., or from Fabri-Kal, Kalamazoo, Mich.

TABLE I. Selection of Test-Dose of Conglutinin.

Comple-ment	Tube method Conglutinin 1:20	1:40	1:80	0	Plate method Conglutinin 1:20	1:40	1:80	0
1: 20	L3	L3	4	0	4	4	4	0
1: 40	4	4	4	0	4	4	4	0
1: 80	4	4	4	0	4	3	2	0
1:160	4	4	2	0	2	±	0	0
1:320	±	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0

L = lysis; 0 = negative; ± to 4 = increasing degrees of conglutination.

volumes of 1.0 and 0.25 ml respectively. After the fixation period, the indicator system (sensitized cells plus conglutinin) was added and the conglutination reaction was allowed to proceed in the incubator at 37°C for 30 minutes, with occasional shaking to insure resuspension of red cells. Antigen and antisera controls, as well as a confirmatory C′ titration were always included in the tests and subjected to the same incubation period as the fixation mixtures. When the conglutinative C′F test was performed in tubes, results were read after centrifugation at 1000 rpm for 1 minute. Tubes were held between thumb and the index and middle fingers of the left hand and repeatedly tapped at the bottom with the index and middle fingers of the right hand to disperse gently the sedimented or conglutinated cells. Unconglutinated erythrocytes formed a homogeneous suspension, while conglutinated cells remained either as a solid disc (4 reaction) or as clumps of varying size (± to 3 reactions). Readings on the plates were also recorded as ± to 4, the last indicating complete conglutination. A gentle circular movement of the plate favors clumping of erythrocytes into the center of the molded chamber and so affords a more clear cut reading. In doubtful cases, readings were repeated 3-5 minutes after complete resuspension of the cells.

Results. a) *Selection of test-dose of conglutinin.* It is well known that amboceptor and C′, as well as C′ and conglutinin are mutually supplementary in conglutination(8). If we desire to titrate C′ or conglutinin, it is then necessary to use maximally sensitized cells in order to remain with only 2 variables (conglutinin and C′), so that one may be titrated with a convenient, arbitrarily chosen dose of the other. Table I summarizes a bloc titration showing this effect. With the exception of the fixation tests carried out with C-reactive protein, all the tests were performed simultaneously with tube and plate methods to check reliability of the latter.

The results obtained with the plate technic were only slightly weaker than those of the tube method in the conglutinin range of 1:20 to 1:80 (Table I). By setting the test-dose of conglutinin at dilution of 1:20, the single batch of horse C′ used here always titrated 1:160 by either the tube or plate technics. In all fixation experiments, conglutinin and horse C′ were used at dilution of 1:20.

b) *Determination of optimal amount of antigen.* Table II gives the results of a bloc titration designed to determine optimal amount of BSA required to measure the C′-fixing potency of a rabbit anti-BSA serum

TABLE II. Determination of Optimal Amount of Antigen.

BSA, 10 μg N/ml	Tube method Rabbit serum anti-BSA 22, 40 μg AbN/ml 1:5	1:10	1:20	1:40	1:80	0	Plate method Rabbit serum anti-BSA 22, 40 μg AbN/ml 1:5	1:10	1:20	1:40	1:80	0
1: 5	0	3	4	4	4	4	0	2	3	4	4	4
1: 10	0	0	3	4	4	4	0	0	1	3	4	4
1: 20	0	0	2	4	4	4	0	0	0	3	4	4
1: 40	0	0	0	4	4	4	0	0	0	0	4	4
1: 80	0	0	0	3	4	4	0	0	0	1	4	4
1:160	0	0	3	4	4	4	0	0	0	2	4	4
1:320	2	3	4	4	4	4	0	2	2	2	4	4
0	4	4	4	4	4	4	4	4	4	4	4	4
Complement titration												
	1:10	1:20	1:40	1:80	1:160		1:10	1:20	1:40	1:80	1:160	
	L1	4	4	4	4		L1	4	4	3	3	

TABLE III. Effect of Fixation Time and Temperature on C′-Fixing Potency of Rabbit Antisera.

	Time and temp. of fixation	Tube method Rabbit anti-BSA No 25	 M	 0	Plate method 25	 M	 0
I.	½ hr, room temp.	10*	10	10	10	10	16
		C′ titer: 160†			C′ titer: 40-80		
II.	1 hr, 37°C	63	25	40	40	25	63
		C′ titer: 80			C′ titer: 20		
III.	20 hr, 0°C	40	40	63	63	40	63
		C′ titer: 80			C′ titer: 40		

* Reciprocal of antiserum dilution required for complete inhibition of conglutination in presence of optimal amount of antigen.

† Reciprocal of C′ dilution giving complete conglutination (4) without serum or antigen, but after same incubation as for fixation mixtures.

(No. 22). Here again, the difference in results obtained with plate and tube technics is negligible. The optimal amount of antigen was either a 1:40 or 1:80 dilution of the stock solution. Deterioration of C′ was slightly greater in the plates than in tubes and this probably accounts for the higher titer given for anti-BSA in the plate (1:40), as compared with titer of 1:20 observed in the tube series.

c) *Effect of fixation time and temperature on CC′F with rabbit antiserum.* It is customary to use a fixation period of ½ hour at room temperature for the CC′F test. In fact, Blomfield, Coombs and Hole(18) have shown that the C′-fixing potencies of anti-mallein sera from different species (horse, cattle, pig, goat, sheep, dog, cat, guinea pig, rabbit, ferret, hamster, rat and human), when assayed by CC′F with horse C′, were only slightly enhanced if incubated overnight at 4°C instead of for ½ hour at room temperature. However, our experiments with rabbit anti-BSA and BSA have shown the former to be preferable (Table III).

As seen in Table III, CC′F titers were considerably higher after 20 hours at 0°C or 1 hour at 37°C than after ½ hour at room temperature. C′ deterioration was about the same when tests were incubated in tubes for 1 hour at 37°C or for 20 hours at 0°C (C′ titer: 80). However, if C′ titrations were carried out in the plate, more deterioration occurred, particularly after 1 hour at 37°C (C′ titer: 20), probably due to deficient protection against evaporation under conditions of the plate test.

d) *Relative titers in hemolytic and conglutinative C′F tests.* Table IV summarizes data which permit a comparison of the fixing potencies of 6 rabbit anti-BSA sera, as measured by the CC′F test (tube and plate technics) with horse C′ and by a refined method of hemolytic C′F using 2 50% units of guinea pig C′. Details concerning the last method will be presented elsewhere. The fixation period for all tests was 20 hours at 0°C.

e) *Use of CC′F in a virus system.* The CC′F plate and tube technics were also used in parallel to demonstrate specific antibodies in sera of guinea pigs hyperimmunized with A and C types of foot-and-mouth disease virus. Fairly good agreement was observed in results obtained with 2 different immune sera and their corresponding antigens. With type A antiserum and its homologous antigen diluted 1/5 and 1/10, antiserum end points (complete fixation) of 1/160 and 1/320 were obtained by the tube and the plate method respectively. Tested under the same conditions, type C antiserum showed the same titer (1/160) by both methods.

f) *Estimation of C-reactive protein in human sera.* The CC′F microtechnic was also used for estimating C-reactive protein in human sera. The results of this study will be reported separately.

Discussion. The results reported here show that the CC′F test may be easily performed on plastic molded plates in a final

TABLE IV. Comparison of Titers* Given by Conglutinative and Hemolytic C′-Fixation.

Rabbit anti-BSA sera diluted to 40 μg AbN/ml	Conglutinative C′F Tube	 Plate	Hemolytic C′F
21	40	63	77
22	63	63	110
24	63	100	137
25	40	63	93
M	40	40	93
0	63	63	200

* CC′F titers are expressed as reciprocals of serum dilutions giving complete inhibition of conglutination; HCF titers, as reciprocals of dilutions, required 50% lysis.

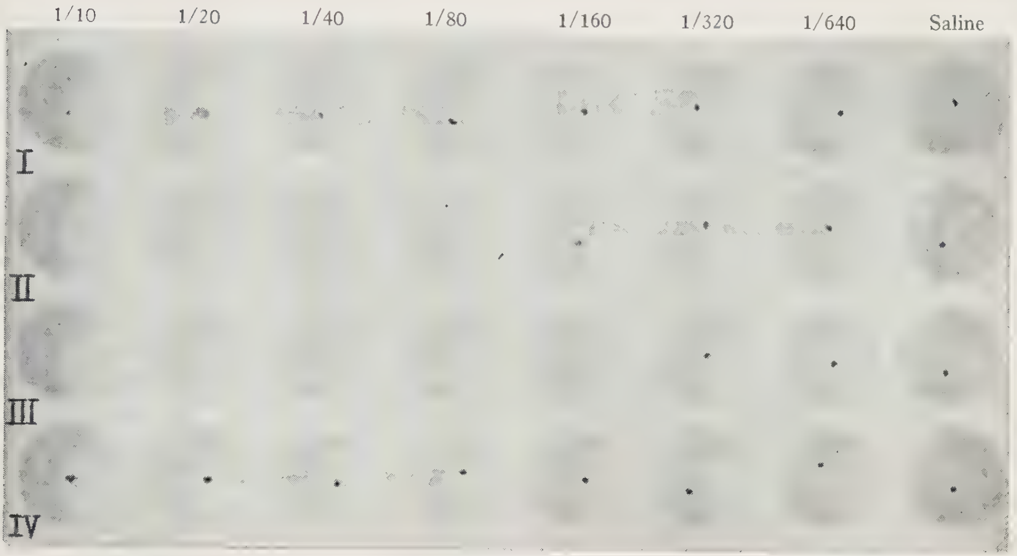

FIG. 1. I—Negative serum. II and III—Complete fixation up to 1/80, partial fixation at 1/160. IV—Saline control

volume of 0.25 ml without any impairment of sensitiveness or specificity. This microtechnic is especially valuable because of the small volumes of reagents used and because of the ease of execution and reading (Fig. 1).

The amounts of antibody N detected by the CC′F microtechnic were in the range of 0.4-1.0 μg/ml for rabbit anti-BSA serum and as little as 0.1 ml of serum suffices for the test, including the serum control. The quantitative information provided by the microtest may be considered as sufficiently accurate for virus typing studies and for estimation of pathological proteins of clinical interest, particularly in cases where only small amounts of material are available.

Summary. 1) A plate technic is described for study of complement fixation with a conglutinative indicator system. The reliability of the new technic was checked by parallel use of the tube technic in experiments with 6 different rabbit anti-bovine serum albumin sera. 2) The conglutinative complement fixation microtechnic has also proved satisfactory for detection of antibodies against foot-and-mouth disease viruses in guinea pig hyperimmune sera, as well as for estimation of C-reactive protein in human sera.

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Toxicity of Pantetheine.* (23212)

Robert P. Knott, Daniel P. N. Tsao, R. S. McCutcheon, Vernon H. Cheldelin and Tsoo E. King
School of Pharmacy, Science Research Institute and Department of Chemistry, Oregon State College, Corvallis.

Pantothenic acid has been shown to prevent achromotrichia in animals. The results, however, are not consistent and very high levels of the vitamin are sometimes required. The metabolically active forms of pantothenic acid are coenzyme A and pantetheine. It is probable that the inconsistency among various experiments may be due to certain uncontrolled factors. For example, rate of formation of pantetheine from pantothenic acid may be limiting in some species.

To study antigray-hair activity of pantetheine, a systematic study of its toxicity is required. As described below, pantetheine is relatively innocuous, with LD_{50} of 5 to 7.5 g/kg body weight of mice.

Methods. The pantetheine was either synthesized in this laboratory(1) or purchased from Krishell Laboratories. Solutions containing 100 to 150 mg of pantetheine and 30 mg glucose/ml in 0.02 M phosphate buffer, pH 6.2, were distributed into 10 ml ampules and sterilized for 30 minutes at 100°C, then stored in a refrigerator. Solutions for control experiments contained all ingredients except pantetheine. In experiments for chronic toxicity, the solutions were diluted 10 fold with sterilized saline solution to obtain a desirable injection volume, about 0.3 ml/animal. Rabbits were kept in individual cages. Mice of both sexes were caged in 2 groups, *i.e.*, control and experimental. Stock diets were used throughout. All animals and diets were kindly supplied from the Animal Husbandry Department of this college.

Results. Two rabbits, weighing 9 and 10 lb, received intravenous daily doses of 0.5 g pantetheine/animal for 4 days. No ill effect was observed.

Acute toxicity and LD_{50} in mice. The LD_{50} test for pantetheine in mice by intraperitoneal injection was conducted as shown in Table I. It is evident that the LD_{50} lies between 5 and 7.5 g pantetheine/kg body weight of mice. At 5 g/kg the mice showed some immediate signs of mild depression but only one animal died. When a 7.5 g/kg dose was administered, 12 animals out of 15 died within 4 hours and another within 20 hours. Two animals survived. General depression and apparent muscular incoordination was observed among most animals. At the 10 g/kg level most animals died within an hour. Within 20 minutes after administration of pantetheine, signs of depression were observed as expected. No such abnormalities were shown among the control animals receiving the same volumes of the solution without pantetheine.

Chronic toxicity. The mice were divided into 2 groups of 51 animals each. Cages were changed 5 times a week after animals were injected and fed. Weighings were made before injection. Two-tenths g of pantetheine/kg body weight was administered during these experiments. Both control and experimental animals received 5 injections each week, Monday through Friday, for 10 weeks. During the first 3 weeks, 5 animals in the control group and 4 in the experimental group died.

TABLE I. Acute Toxicity of Pantetheine in Mice.

Dose, g/kg*	Avg vol of inj., ml	Avg individual wt of mice, g	No. of animals tested	No. of animals died (24 hr)
2	.32	16	13	0†
5	.97	19.5	13	1
7.5	.94	18	15	13
10	1.18	17.2	12	12
0	.35	17.9	11	0†
0	1.39	18.4	11	0†

* In g of pantetheine/kg body wt.
† No death after 1 wk.

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After that time, no deaths were observed. The controls appeared to have a higher average mean weight, namely about 2%, than did the experimental animals based on the first weighing.

However, the experimental group contained 29 females and 22 males whereas the control group contained 23 females and 28 males. The apparent higher gain in body weight in the control group could be attributed to unequal sex distribution in these 2 groups. Thus the *ratio* of mean weight of the experimental animals to that of the controls was calculated and was practically constant at about 0.96. The increase in ratio from the 44th injection onward was due to pregnancy of the animals since more females were in the experimental group. It is concluded that administration of pantetheine at 0.2 g/kg did not affect growth of mice under the conditions tested.

Summary. The LD_{50} of pantetheine in mice was found to lie between 5 and 7.5 g/kg body weight. General depression, and eventual death were observed in animals after administration of high dosages of the compound. Intraperitoneal injections of pantetheine at 0.2 g/kg body weight continuously for 10 weeks did not affect growth of mice.

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Amylase Clearance and Excretion During Water Diuresis.* (23213)

ROBERT L. MCGEACHIN AND LILA A. HARGAN
Department of Biochemistry, University of Louisville School of Medicine, Ky.

The effect of increasing urinary flow on urea clearance values(1,2) has been well established. Urea clearances rise rapidly at low urine flows up to 0.35 ml/min. but above this rate of urine flow the increase in clearance is slight. Inulin and PAH clearances in man(1,3) do not seem to be influenced by diuresis. However, data on effect of diuresis on protein excretion and clearance are lacking. In our previous study(4), amylase clearances in man with normal urine flow were determined and found to fall in the range, 1-3 ml/min. but the effect on amylase excretion and clearance of increasing urinary flow to high levels was neither determined experimentally nor could it be accurately predicted from an examination of the plot of amylase clearance *vs.* urine flow for the data available. Data on amylase clearances at high urine flows produced by water diuresis have now been obtained and are described here.

Methods. The human subjects were 10 male and 3 female medical students, ages 22-29. The subjects were instructed to drink water freely during afternoon and evening of the days prior to their individual clearance studies since the studies were carried out during a period of hot weather, July and August 1956. To produce diuresis, each subject drank 1500 ml of water in 3 equal portions at half hour intervals prior to beginning of urine collection period. At zero time, the bladders were emptied and the last 500 ml of water taken. Approximately one hour later (time accurately recorded) the bladders were again emptied and these urine specimens collected. At approximately the mid-point of the intervening hour, blood samples were drawn by venipuncture for serum amylase determinations. Both urinary and serum amylase levels were determined by Van Loon's(5) method. Clearances were calculated using the standard formula $\frac{UV}{P}$ = clearance, where U = urine concentration, P = plasma or serum concentration and V = urine flow in ml per minute. All values for urine flow, amylase clearance and amylase excretion are corrected to 1.73 square meters of body surface. The diuresis produced such dilute urine that urinary amy-

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TABLE I. Amylase Clearance during Water Diuresis in Normal Human Subjects.

Subject	Urine vol, ml/min.*	Urine amylase, units/100 ml	Amylase excreted, units/min.*	Serum amylase, units/100 ml	Clearance, ml/min.*
♂	15.6	15.5	2.42	146	1.67
	3.2	83.2	2.66	83	3.22
	15.4	12.3	1.89	55	3.43
	15.1	9.8	1.48	42	3.54
	15.3	12.8	1.96	43	4.57
	12.0	13.6	1.63	71	2.29
	3.4	17.6	.60	53	1.13
	13.3	10.0	1.33	40	3.32
	18.6	11.4	2.12	79	2.68
	11.9	13.2	1.57	73	2.17
	Mean		1.77		2.80 ± .29
♀	15.2	11.4	1.73	49	3.55
	6.4	26.3	1.68	69	2.41
	9.8	10.3	1.01	76	1.33
	Mean		1.47		2.43
	Mean for both groups		1.70		2.72 ± .28

* All values are corrected to 1.73 m^2 of body surface.

lase concentrations were too low to measure accurately in the usual 1:10 dilutions with isotonic saline. Instead, the chloride concentrations were made up to 0.16 M by addition of solid sodium chloride to the undiluted urines and these used directly in the amylase analyses. Thus the undiluted urine samples, with amylase contents of 10-20 units/100 ml, were about the equivalents of 1:10 dilutions of human serum.

Results. Comparison of amylase clearance values during the diuresis (Table I) with those(4) obtained at urine flows of 3 ml/min. or less (these clearances were 2.08 ± 0.18 ml/min. for 13 men and 1.52 ± 0.15 for 12 women) indicates that amylase clearance increases only slightly during diuresis. Comparing only the groups of men in the 2 experiments since there are only 3 women in the diuresis tests, the difference $(2.80 - 2.08 = 0.72)$ in mean clearances is of only borderline statistical significance(6): $t = 0.72/0.346 = 2.08$. For 21 degrees of freedom, $t\ (0.05) = 2.08$.

Comparing rates of amylase excretion per minute, a mean of 1.65 units/min. was found for all subjects (1.78 for men) at low urine flows and 1.70 units/min. for all subjects (1.77 for men) during diuresis. The percentage difference in excretion rates is much less than that for the clearance values and is not statistically significant.

In one subject (M8), amylase clearance was determined in three 20 minute intervals and was constant throughout the entire hour. Successive values were 3.26, 3.34 and 3.36 ml/min. for the 3 periods at a serum amylase level of 40. In the same individual, redetermination of amylase clearance without diuresis at a urine flow of 0.93 ml/min. gave a clearance value of 3.12 ml/min. at a serum amylase level of 46. In 7 other subjects for whom clearance data at both high and low urine flows was available, the clearances were not necessarily as constant, agreeing fairly well only when the serum amylase levels were about the same in the two determinations. Mean values at high and low urine flows were 66 and 95 units/100 ml for serum amylase, 1.59 and 1.63 units/min. for amylase excretion and 2.54 and 1.93 ml/min. for amylase clearance.

Discussion. The data presented indicate that amylase clearance increases only very slightly with increasing urine flow and that amylase excretion remains constant over a wide range of urine flow. The slight increases in clearance may be due to increased glomerular filtration rates. While it might appear from the data that amylase excretion is independent of serum amylase levels within the normal range, this is not a likely possibility. The observation that a plot of urine amylase concentration *vs.* urine flow resembles a rectangular hyperbola(4) would fit this picture but other observations(7) show that excretion does increase with increasing serum levels, reaching values of 50-70 units/min. in patients with acute pancreatitis.

It was noted that 6 of the group subjected to water diuresis had sub-normal serum amylase levels at the time blood samples were drawn. The mean serum amylase level for the whole group was 68 as compared to 99 for the group with low urine flows. While it is not suggested that the diuresis was responsible for the low serum amylase levels, the fact that low levels did prevail may be the reason that clearances were higher in this group than in the one with low urine flows. Clearance values would be greater at subnormal serum levels if amylase excretion remained essentially unchanged.

The data in these experiments again seem to indicate that amylase is filtered by the glomeruli but not reabsorbed in the tubules. No indication of a threshold is seen and clearance values do not increase with increasing serum levels as would be the case if reabsorption were at all appreciable. If there is no reabsorption of amylase, one would then not expect increasing urine flow, at which time there is a decreased reabsorption of water, to influence amylase excretion or clearance.

Summary. 1. Amylase clearance increases only slightly during water diuresis. 2. Amylase excretion remains constant during water diuresis.

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A Freely Rotating Adapter for Cardiac Catheterization. (23214)

Robert Rosenblum[*] (Introduced by R. E. Weston)
Medical Division, Montefiore Hospital, New York City.

The rotation of a cardiac catheter for the purpose of directing the tip to a desired position frequently results in marked torsion and occasional breakage of the proximal end of the catheter and, at times, spontaneous disconnection of the catheter from the stopcock. Either the deliberate separation of the catheter to release the torsion, or spontaneous disconnection, may allow air to enter the system, disrupt the continuous flow of anti-coagulant fluid, and prevent proper pressure recording.

Methods. To facilitate free and continued manipulation of the cardiac catheter without allowing separation of the infustion apparatus, a Luer-Lok adapter with a sleeve allowing free rotation in either direction has been developed. The adapter is made of nickel or chrome plated brass with a rotating Luer-Lok section connected to the catheter (Fig. 1). The external rotating ring is larger than the rest of the adapter to allow for better gripping (Fig. 2A). The adapter can be unthreaded for more efficient cleaning and also to release tension of the inner rotating ring if it becomes too stiff (Fig. 2B and Fig. 3). The opposite end of the adapter is a conventional female unit (Fig. 2C). The inner bore is .073″ diameter. The O ring is rubber[†] and has a temperature range of −40 to +300°F. The durometer of this ring is 70 shore "A" (±5). The O ring is the inner part of the larger rotating metal ring. Fig. 3 shows cross sectional details. The adapter and the stopcock have been also made into one unit using a standard B-D 5031 MS type 3 way stopcock with an inner bore of .073″

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[*] Research Fellow Amer. Heart Assn.

[†] Purchased from Precision Rubber Products Corp.

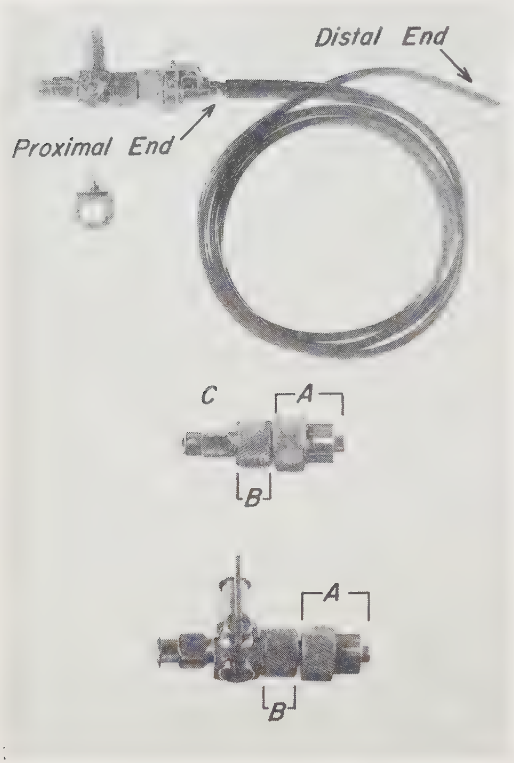

FIG. 1 (top). Catheter attached and locked to rotating adapter stopcock unit. Profile view of clamp.
FIG. 2 (bottom). External view of rotating adapter units.

in diameter (Fig. 2, 3). With the usual catheters of larger diameters, rotation at site of cutdown is transmitted with enough torsion to the proximal end that the adapter may rotate spontaneously and therefore release tension automatically. With smaller catheters or less rigid catheters the adapter can be used either to prevent or relieve torsion: The former by rotating the catheter at the site of the cutdown simultaneously with the adapter, or the latter by rotating the adapter after rotation of the catheter. This adapter unit provides no advantage when double lumen catheters are employed.

Because the catheter is attached to the Luer-Lok of the adapter with a clockwise twist, a counter-clockwise rotation theoretically may detach the catheter from the adapter, although, in our experience during catheterization, this has not occurred. To avoid this potential complication, a small clamp has been devised which holds the 2 units together during rotation. The clamp is a chrome plated brass 2 pronged bar attached to a ring with a thumbscrew. The ring fits over the adapter; the prongs fit over the catheter hub. One can lock the 2 units together by tightening the ring thumbscrew (Fig. 1).

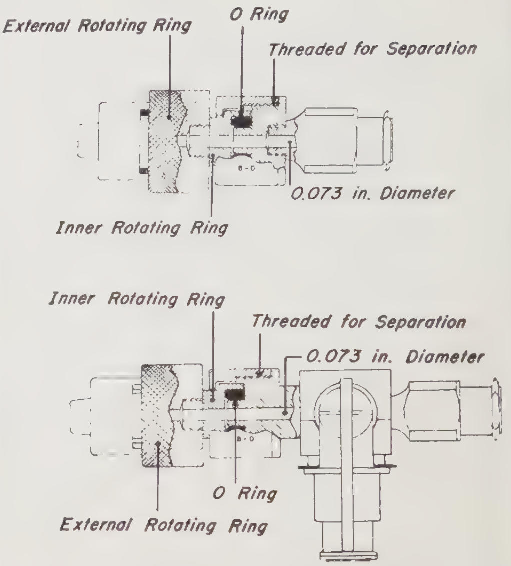

FIG. 3. Cross-section blueprints of rotating adapter and rotating adapter stopcock unit.

The rotating adapter has functioned satisfactorily and remained leakproof after frequent sterilizations, autoclavings, innumerable rotations and subjected to tests with greater negative and positive pressures than are encountered during catheterizations. It may have wider application wherever one desires tubing to have free rotation without torsion.

Summary. A freely rotating adapter and clamp for the connection of the cardiac catheter to recording and perfusion apparatus is described.‡

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‡ Since this manuscript was submitted, a paper by Gerish, R. A., and Dodrill, D. F., *Harper Hosp. Bull.*, 1952, v10, 133, describing a rotating syringe was called to the author's attention. However, it is felt that the present rotating adapter represents a distinct improvement over this device.

The author thanks James Buck of Becton-Dickinson Co. for his cooperation in the development and manufacture of this adapter. He also acknowledges helpful suggestions of Dr. Doris J. W. Escher, of Cardiac Catheterization Group, in developing the adapter and preparing the manuscript.

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Early Clinical Evaluation of a New Oral Non-Sulfonylurea Hypoglycemic Agent.* (23215)

LEO P. KRALL AND RAFAEL CAMERINI-DAVALOS (Introduced by Alexander Marble)
Joslin Clinic and Baker Clinic Research Laboratory, Boston, Mass.

Recent medical literature has contained many reports of new, oral hypoglycemic agents(1,2,3). These seem to have hypoglycemic effect in certain types of diabetics, but are sulfonylurea derivatives and as such may have certain limitations. Recently Ungar *et al.*(4) reported that a new, oral non-sulfonylurea hypoglycemic agent was effective in both normal and alloxan-diabetic animals. Pomeranze(5) reported successful hypoglycemic action in early clinical trials.

This report is an early clinical evaluation of the hydrochloride of N^1beta-phenethylformamidinylimino urea, referred to hereafter as DBI. The object of this study was to determine whether DBI has blood sugar lowering properties and whether there are any immediately discernible or early toxic effects.

Materials and methods. Twenty known, proven or newly diagnosed diabetics were studied for periods varying from 2 days to 3 months. They were first hospitalized and given a constant, weighed diet. The patients were checked separately by 3 different observers at least twice daily. Previously known diabetics receiving insulin had their insulin discontinued and DBI substituted as needed. New diabetics were treated with DBI after the diagnosis was established. The laboratory studies consisted of venous, fasting, Somogyi-Nelson(6) blood sugar and capillary blood sugar determinations at varying intervals throughout the day. Urine sugars were determined by the Benedict method, fasting and at 11 a. m., 4 p. m. and 9 p. m. daily. A 24-hour total urine sugar was also done. Each patient had a white blood count, blood hemoglobin and platelet determination, as well as a complete urinalysis. Some patients had complete liver function tests which included sulfobromophthalein, thymol turbidity, alkaline phosphatase, cephalin flocculation and blood bilirubin studies. The 20 patients included 8 males and 12 females, whose ages varied from 34 to 80 and averaged 61 years. The duration of diabetes ranged from 1 to 33 years and averaged 18 years. Twelve of the 20 patients took insulin previously and their doses varied from 6 to 58 units daily. The average insulin dose was 28 units daily. After the blood sugar lowering ability was ascertained, the group was classified into one of 3 categories: (1) successful; (2) partially effective and (3) ineffective. Class I, Successful—indicated definite blood sugar lowering effect and included (a) those patients controlled on an adequate insulin dosage and weighed diet who were able to maintain the same degree of control with DBI plus weighed diet; (b) those with elevated blood sugars, previously uncontrolled, who showed a definite drop in blood sugar level on DBI plus weighed diet. Class II, Partially Effective—included those with a definite blood sugar lowering effect who were unable to tolerate effective doses because of side effect severity. Class III, Ineffective—included patients with no significant change in blood sugar level in spite of at least 2 days' administration of 150 mg or more of DBI daily.

Results. There was a demonstrable blood sugar lowering effect in 13 of the 20 patients studied (Fig. 1 and 2). Three others were classified as partially effective since the blood sugar lowering effect was apparent, although DBI was discontinued because of severe side effects. The patients designated as successful demonstrated a definite decrease in blood sugar levels along with diminished glycosuria and disappearance of 24-hour urine glucose output. With those patients continued on DBI for prolonged periods, discontinuing the DBI or substituting a placebo resulted in a return to elevated blood sugar levels. There were no hypoglycemic reactions observed. The maximum daily requirement of DBI was

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* We are indebted to Dr. L. Freedman, U. S. Vitamin Corp., N. Y. City, for the DBI used.

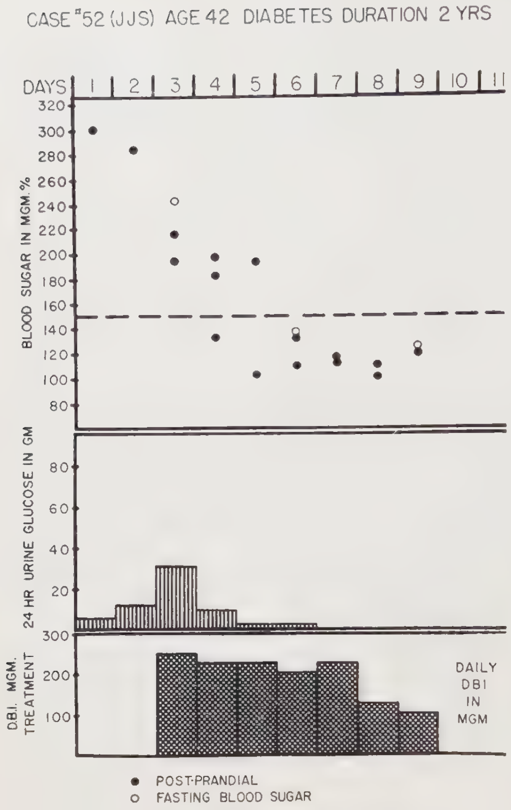

FIG. 1.

from 50 to 250 mg in divided doses. The first effective dose which lowered the blood sugar varied from 50 to 200 mg.

Thirteen of the group showed no side effects of any kind. The other 7 had side effects from slight to severe. These included 5 with nausea, 4 with diarrhea, 2 with vomiting and 2 with dizziness probably secondary to the vomiting. Three of the cases were uncomfortable enough to warrant discontinuing the drug. The symptoms improved within a few hours after the drug was stopped. In 2 of the 4 failures, the drug was ineffective in spite of daily doses of 200 and 225 mg. In the other 2 cases, the doses were insufficient because of side effects which appeared before the effective level had been reached. Except for these side effects, no other untoward findings or toxic manifestations have been found to date. The patient with the longest period of treatment has now received DBI 3 months.

Discussion. If it is true that DBI increases glucose uptake in isolated rat diaphragm and that it is fully active in alloxan-diabetic animals, this drug may have a potential of considerable significance, providing no toxic manifestations develop. At present the incidence of side effects is too high, but improved technics of administration or possible chemical changes might alleviate this difficulty. It is interesting to note that as yet the side effects seem to be limited to the gastrointestinal tract and have been observed even in the presence of high blood sugar levels. This suggests that the annoying symptoms are not related to hypoglycemia. Further study is needed to find range of effectiveness and to ascertain any toxic effects. The doses effective in this study are much smaller than those required to lower blood sugar levels in the reports of other oral hypoglycemic agents.

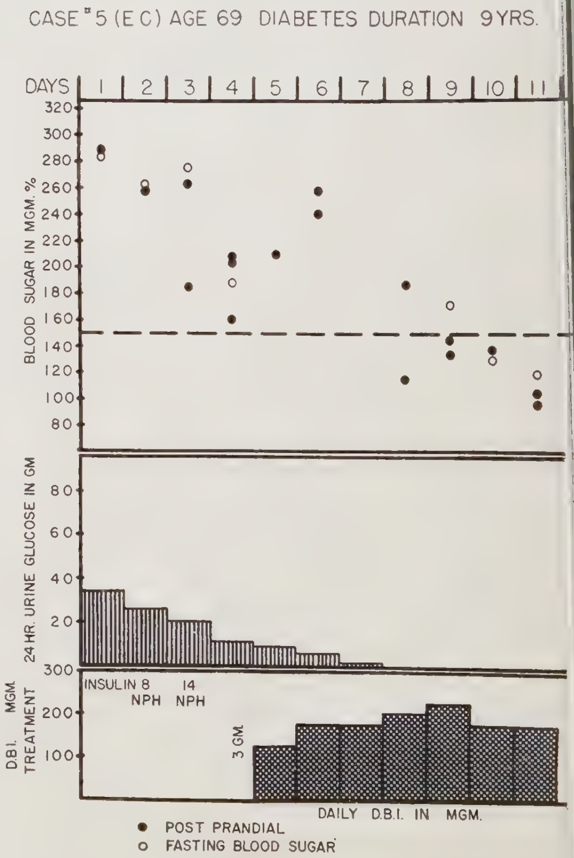

FIG. 2.

Summary. 1. This is an early evaluation of a new, non-sulfonylurea hypoglycemic agent. 2. In this study of 20 cases, DBI, the hydrochloride of N^1beta-phenethylformamidinylimino urea, appears to have definite blood sugar lowering properties. The mechanism of action is not yet known. 3. There are annoying side effects in some cases which are restricted to the gastrointestinal tract and are not related to the blood sugar levels. 4. No other annoying or toxic effects have been observed at this time. 5. The size of the effective blood sugar lowering dose is smaller than that reported for other oral hypoglycemic agents.

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Desoxyribonucleic Acid (DNA) Content of Mammary Gland During Pregnancy and Lactation.*† (23216)

David R. Griffith and Charles W. Turner
Department of Dairy Husbandry, University of Missouri, Columbia.

It has been postulated that for any given species the somatic cell nuclei contain a constant amount of desoxyribonucleic acid (DNA)(1). This being true, determination of DNA would provide an accurate estimation of the number of cells formed during the developmental phase of a tissue, gland or organ(2). Upon the basis of this theory, studies have been conducted to determine total DNA of mammary glands of rats(3), rabbits(4) and mice(5) during normal and experimental development of the mammary gland in order to measure extent of mammary gland growth.

It was thought necessary to provide a sound basis for the above studies(3,4,5) by determining the DNA content per cell of mammary gland during growth and lactation.

Methods. Virgin, pregnant and lactating primiparous albino rats were used. Pregnancy ranged from 15-20 days and was determined by examination of fetuses. Animals which were used for determination of DNA of early lactation were all taken on the day of parturition. The days of late lactation ranged from the 18th to the 21st. The animals were killed and the 6 posterior glands were quickly removed and placed in 30 ml of ice cold isotonic sucrose containing 1% citric acid. The kidney, spleen and mammary gland were treated in an identical manner.

Tissue was homogenized for 2 minutes in a Waring Blendor(6) and strained through cheesecloth, 2 and 4 layers in thickness. This filtered homogenate was then used for subsequent fractionations. Filtered homogenate was centrifuged 30 minutes at 2100 rpm, 1100 rpm for 15 minutes, 800 rpm for 10 minutes and 600 rpm for 10 minutes. Final sediment was suspended in 10 ml of homogenizing solution and nuclei were then counted on a hemocytometer. Tissue residue was extracted with ice cold 10% trichloracetic acid and DNA determined by a method recently published(7). DNA per cell was then determined by dividing total quantity of DNA by known number of nuclei in the original suspension.

Results. It was observed that DNA of the nucleus remained within reported literature values in the virgin and the pregnant animal. In the virgin animal DNA content of pooled

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* Contribution from Department of Dairy Husbandry, Mo. Agr. Exp. Sta. Journal Series No. 1736. Approved by Director.

† This investigation was supported in part by grant from Am. Cancer Soc.

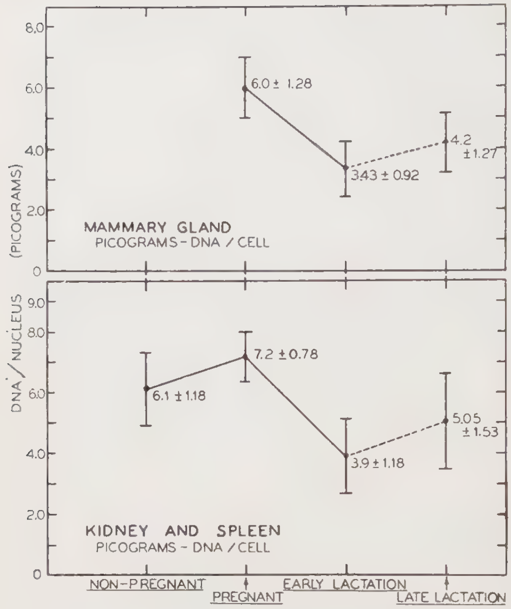

FIG. 1. DNA content per nucleus of mammary gland cells of rat during pregnancy and lactation compared to kidney and spleen cells. The marked decline in DNA per cell in early lactation has not been reported previously.

kidney and spleen only was determined due to insufficient development of the mammary gland at this stage. DNA per nucleus in virgin animals had a mean average of 6.1 picograms,† while the mean for pregnant animals was 7.2 for pooled tissue and 6 picograms for mammary gland.

On the first day of lactation, in both tissues there was an appreciable drop in nuclear DNA content. The mean value for pooled tissue was 3.9 picograms and for mammary gland 3.4 picograms. There was a statistical difference ($P < .0025$) between the pregnant animal and the animal in early lactation.

By the time of late lactation DNA had risen to the mean value of 5.05 picograms for pooled tissue and 4.2 for mammary gland. These values were not statistically different from those obtained during the non-pregnant and pregnant stages of this study.

Throughout the experiment there was no statistical difference among groups with the exception ($P < .05$) of pooled kidney and spleen and mammary gland during pregnancy.

A number of determinations were carried out on male mice with experimentally induced mammary gland growth. The values, an average of 5.1 picograms per nucleus, were within the reported value of other somatic tissues of the mouse (kidney and spleen) of 5 picograms per nucleus(8).

Discussion. The results of this experiment show that during the period of pregnancy or experimentally induced gland growth the estimation of DNA would be an accurate measurement of cell multiplication. The abrupt drop of DNA per cell at onset of lactation seems to be contrary to reports in other tissues. To the authors' knowledge, this is the first report of DNA content of cells during the physiological changes of pregnancy and lactation. The estimation of cell number during this period would be accurate only if a correction factor were applied for the change in DNA.

Summary. A study of the DNA concentration per nucleus was carried out on kidney and spleen and the developing and lactating mammary glands of rats. The DNA concentrations remained within normal values in the pregnant and non-pregnant stages. On the first day of lactation there was an abrupt drop in this value which returned approximately to normal by 18th day of lactation.

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† 1 picogram (pg) = 10^{-12} g.

Intramuscular Administration of Digoxin in Propylene Glycol.* (23217)

Aaron Ganz,† Hiroshi Fujimori,‡ Mario Penna,§ Theodore Greiner|| and Harry Gold

Department of Pharmacology, Cornell University Medical College, Beth Israel Hospital, and Hospital for Joint Diseases, N. Y. City.

Injection of common digitalis glycosides by the intramuscular route has been restricted by two limiting factors: Many of the glycosides dissolve poorly in water, while solvents that are effective also irritate the site of injection. Experience with propylene glycol as a vehicle for intramuscular injection(1,2) led to its trial with the digitalis glycosides. The preparation described in this report contained digoxin, 0.25 mg per cc, in 40% propylene glycol and 10% ethyl alcohol.¶ Onset and persistence of cardiac action were compared after injection by the intravenous and by the intramuscular route. Local reactions were examined after injections of intramuscular digoxin, and after injections of the solvent.

Method. Cardiac effect was measured in patients with auricular fibrillation, as described in detail elsewhere(3). Seven patients who maintained rapid ventricular rates without digitalis were placed at complete bed rest in the hospital. The etiology of their heart disease varied, their ages ranged from 34 to 75 years, and 4 were female. None had gross edema. After one or 2 weeks, when the apex rate had declined to a constant level, an injection of digoxin was given and the rate followed closely. In another week or 10 days the second injection of digoxin was given by the alternate route. In every case the dose was 1.2 mg, the full digitalizing dose of digoxin(3). Each patient received an intramuscular and an intravenous dose, the order being reversed in 3 of the 7 subjects. Additional injections were made for systematic study of the local reactions by a method previously described(4). Each of 26 ambulant patients not on digitalis received an injection deep into the buttock muscle, and a week later a second injection into the opposite buttock. One injection was 2 cc of the digoxin solution, the other 2 cc propylene glycol, and the sequence random. The physician who questioned them and examined the site did not know which material had been injected.

Results. The curves in Fig. 1 demonstrate the more gradual onset of digoxin effects following intramuscular injection. At 1 hour, when the activity of intravenous digoxin was virtually complete, that of intramuscular digoxin was just perceptible. Maximum effect reached the same intensity for the 2 routes, though present by 2 hours after intravenous injection and not until 8 hours after intramuscular injection. The apex beat returned toward control level at the same rate whether the injection was intramuscular or intravenous.

Variability in the effect was studied from

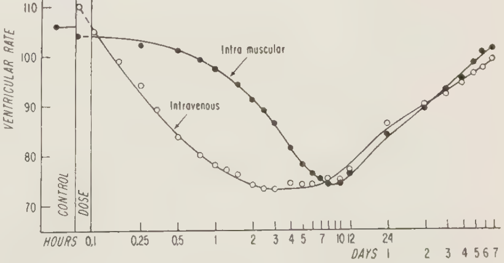

FIG. 1. Development and persistence of cardiac action in 7 patients with auricular fibrillation. Horizontal bars in space labeled "control", are average rates after one to 2 weeks of bed rest. "Dose" represents 6 minutes for intravenous injection, after which the logarithmic scale begins.

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* Supported by research grant form Nat. Heart Inst., and Digitalis Fund of Cornell University Medical College.

† Research Fellow of N.I.H.

‡ Present address, Tokushima Medical College, Tokushima, Japan.

§ Kellogg Foundation Fellow from University of Chile.

|| Present address, Baylor University College of Medicine.

¶ "Lanoxin" brand digoxin, supplied by Burroughs Wellcome & Co.

TABLE I. Local Reactions to Intramuscular Injection.

% of 26 patients with	Time of examination				
		Days			
	1 hr	1	2	3	7
	Digoxin preparation				
Severe pain	50	15	0	0	0
Erythema	0	8	12	8	0
Induration	0	42	4	4	0
	Propylene glycol				
Severe pain	4	0	0	0	0
Erythema	0	0	0	0	0
Induration	0	23	8	4	4

each of the 14 individual ventricular rate curves. The deepest depression of rate sustained for at least 3 hours was read from each curve. That depression fell lower after intravenous injection in 4 patients, after intramuscular in 3. The mean effects for the routes were not different; standard errors were 2.5 beats per minute for intravenous and 3.6 for intramuscular. No systemic toxicity was encountered.

Table I summarizes the local reactions and their time of onset. Within 1 hour of intramuscular digoxin half of the patients developed severe pain, and most of these showed tissue reactions the following day. In the parallel test, propylene glycol caused little pain and less frequent tissue response. Twenty additional patients served in a final "double-blind" comparison between reactions to the solvent (40% propylene glycol and 10% ethanol) and to physiological saline. In these 20 patients there was neither tissue reaction nor severe pain, although 9 of them mentioned fleeting pain after the solvent and 3 did so after saline.

Discussion. Absorption from an intramuscular depot of digoxin is not fast enough to serve an emergency that demands rapid digitalization. This preparation might act as a convenient substitute for oral digoxin in patients temporarily unable to take regular digoxin tablets. Since only two-thirds of the activity of oral digoxin is detected on the heart(3), a shift from oral to intramuscular digoxin should include reduction in dosage.

Summary. Digoxin in 40% propylene glycol and 10% ethanol was injected intramuscularly in 7 patients with auricular fibrillation. Its full cardiac effect was reached in 8 hours, while intravenous digoxin in the same patients reached full effect in 2 hours. Intensity and persistence of action were the same for both routes. Additional intramuscular injections in 26 patients revealed pain but no permanent tissue damage.

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Intestinal Absorption of Pyrimidines in the Rat.* (23218)

GEORGE RENDINA, E. J. SARCIONE, C. J. LEE, AND H. W. BARRETT
Department of Biochemistry, University of Kansas School of Medicine, Lawrence.

Continuing interest in the metabolism and pharmacology of pyrimidines and pyrimidine derivatives(1,2) prompted us to make a study of the intestinal absorption of a number of 2-thiopyrimidines and of 3 natural pyrimidines. Compounds of this type have frequently been studied by oral administration to animals, although it has not been determined which of them is adequately absorbed from the gastrointestinal tract. We wished to obtain information on this point, and to determine whether any consistent changes in absorption were produced by various substituents in the ring. Since a number of 2-thiopyrimidines have antithyroid activity, it was possible in some instances to correlate rate of absorption with presence or absence of antithyroid activity.

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* Supported by research grants from the Public Health Service, and from the Smith, Kline, and French Foundation. Abstracted in part from M. A. thesis of George Rendina.

Procedures. Sprague-Dawley rats, fasted 24 hours, and weighing 200-250 g were used for all tests. Compounds were prepared as 5 mM solutions in 0.154 M phosphate buffer at pH 6.6, and 2 ml, containing 10 μM of compound (*e.g.,* 1.28 mg thiouracil), administered intragastrically by means of a #10 soft rubber catheter attached to a hypodermic syringe. Although 6.6 is considered to be the normal average pH of the rat intestine(3), it was necessary to test a few compounds at pH 8 because of their poor solubility at pH 6.6. After 30 minutes, the test animal was killed, the stomach and small intestine removed and washed out with enough phosphate buffer at pH 6.6 to give a total volume of 50 ml. The combined washings were filtered, and recovered thiopyrimidine determined colorimetrically with Grote reagent(4). Natural pyrimidines were determined by the lithium arseno-tungstate method of Soodak *et al.*(5). Since phosphate interferes in this test, physiological saline was used for washing out the tract. All readings were corrected for blank, and the fraction of dosage not recovered was calculated as % disappeared. Disappearance by loss into the cecum was excluded since administered compounds seldom traversed more than ⅔ the length of the intestine in 30 minutes. Disappearance by enzymatic destruction prior to absorption was also excluded, since incubation tests *in vitro* showed that digestive enzymes do not attack pyrimidines. Adsorption of pyrimidines on proteins does occur at the normal pH of the intestine(6), but attempts to recover adsorbed material by acid washes did not give good results. An estimate of adsorption was obtained by placing 2 ml of pyrimidine solution in the duodenum of the intestine from a freshly-killed rat, and washing through immediately. Recovered compound was determined as before, and the % adsorption calculated. The difference between % disappeared and % adsorbed was taken as % absorbed. When the animal was anesthetized and the stomach ligated at the pyloric end, administered pyrimidine was recovered almost completely. Hence, the stomach was not used in measuring adsorption, since any material bound in this way was apparently removed by acidic secretions during the 30-minute disappearance tests. *Antithyroid tests.* Thiopyrimidines were prepared as olive oil mulls, and 50 μM/day injected subcutaneously into 30-day-old Sprague-Dawley rats. After 6 days, 5 μC of I^{131} sodium iodide was injected intraperitoneally, and the 24-hour accumulation of isotope in the thyroids measured. In other tests, thiopyrimidines were injected for 13 days, then the thyroids analyzed for total iodine.

TABLE I. Intestinal Absorption and Adsorption of Pyrimidines in Rats.

Derivative	No. of animals	% absorbed		% adsorbed	Oral antithyroid activity in rats(7)
(Thiouracil)	14	41	2.2	23	1
5-Methyl	5	41	3.5	27	.7
6-Methyl	8	41	2.6	22	1
5-Propyl	5	40	4.0	33	2
5-Iodo	9	38	3.0	35	.5 *
3-Methyl	9	33	2.2	33	.5
6-Propyl	10	30	1.6	33	11
5,6-Dihydro	5	43	3.5	17	0
5-Carboxy	10	20	2.0	16	0 †
4-Amino	7	16	3.8	31	.001
6-Carboxy	10	11	1.2	24	.001
4-Amino-5-carboxy	8	0		25	0
Thiobarbituric acid	10	4	.9	25	.003
6-Methyl-5-iodo‡	9	47	2.4	32	0 *
1,6-Dimethyl‡	10	40	1.5	22	.3 †
6-Butyl‡	9	36	1.8	32	3
5-Cyano‡	15	9	3.9	26	.001
Uracil	6	Complete		67	
Cytosine	5	46	2.1	10	
Orotic acid	5	19	2.6	10	

* By thyroid iodine analysis(8).
† Determined for this paper.
‡ At pH 8.

Results. Table I shows the results of the absorption studies as % absorbed ± mean standard error, and as % adsorbed. These figures may be converted directly to micromols by dividing by 10. Thiopyrimidines are named as derivatives of 2-thiouracil, and the ring is numbered in accord with IUC nomen-

TABLE II. Antithyroid Activity of Injected Thiopyrimidines.

Thiouracil derivative	Thyroid I_2, µg/100 mg	Antithyroid rating	Thyroid I^{131}, 24-hr, % of dose*	Antithyroid rating
Controls	68		12.3	
(Thiouracil)	15	1	3.28	1
3-Methyl	12	1	1.31	2.5
5-Carboxy	27	.55		
1,6-Dimethyl	32	.5	5.22	.6
6-Methyl-5-iodo	35	.43	.79	4
5-Cyano	55			
Thiobarbituric	53			
4-Amino	60			
4-Amino-5-carboxy	58		8.17	.4
5-Iodo	33	.5	.67	5
6-Carboxy			10.8	
5,6-Dihydro	70			

* Probable error of counting <5%.

clature. The number of animals refers to the absorption figure. Adsorption was measured with 3-5 animals, and all measurements were within 3% of the mean. In addition to the compounds shown in the Table, thiobarbituric acid and the carboxy derivatives were also tested at pH 8, and showed some improvement in absorption. Absorption of uracil was so rapid that none could be recovered from the intestine after 30 minutes even when the dosage was increased to 15 µM.

Results of the antithyroid studies are shown in Table II. Ten animals were used in each group, and an antithyroid rating calculated when the mean difference from the controls was significant at the 5% level or less. In previous work, the 6-methyl-5-iodo derivative was found to be inactive as an oral antithyroid compound(8). Present results indicate that the oral inactivity was due to low solubility, and consequent poor absorption, at the normal pH of the intestine.

Discussion. With the possible exception of uracil, intestinal absorption of the pyrimidines studied was undoubtedly passive. Very small quantities were absorbed in 30 minutes (*e.g.*, 0.52 mg thiouracil), and absorption of 4 different thiopyrimidines was unchanged when the solutions were made 10^{-3} M in phlorhizin. The pyrimidines most poorly absorbed are fairly strong organic acids, and extensively ionized at pH 6.6. Improved absorption of these compounds at pH 8 was most probably due to increased solubility, since they are difficultly soluble in neutral aqueous media, and may have partly precipitated in the stomach when administered at the lower pH. Aside from strongly ionic groups, comparison of different derivatives shows that any substituent that increased polarity of the molecule decreased intestinal absorption, while non-polar substituents (small alkyl groups) had no effect so long as the molecule remained soluble at the pH used. The position of the substituent in the ring was relatively unimportant.

It has been reported that intestinal absorption of both D- and L-amino acids is also inhibited by ionization or by presence of polar side-chains(9,10) despite active absorption of the L-isomers. Further examples of this same relationship as reported in the general biological literature(11) suggest that in any series of similar, water-soluble compounds, the rate of intestinal absorption is inversely related to polarity. Since strongly polar thiopyrimidines generally lacked antithyroid properties when injected, polarity may also inhibit penetration of compounds into the thyroid. Without further information on disposition of these compounds in other tissues, this possibility is only speculative. However, the results convince us that 6-butylthiouracil for instance, is indeed slowly absorbed from the intestine because of low solubility, but exhibits antithyroid potency because of favorable handling in the tissues.

Summary. The intestinal absorption of 3 natural pyrimidines and of 17 2-thiopyrimidines was studied in intact rats, and the antithyroid activity of 12 of the latter compounds tested by subcutaneous injection. With the possible exception of uracil, all compounds tested were passively absorbed. No relation was found between intestinal absorption and adsorption, and among compounds showing oral antithyroid activity, no relation was found between this activity and absorption. Both absorption and antithyroid activity were decreased by ionization or by polar groups, while small, non-polar groups had little or no effect on absorption. The position of the groups was of minor importance. It is suggested that the rate of intestinal absorption, for any series of similar, water-soluble compounds, is inversely related to polarity.

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Effect of Distillers Dried Solubles and Molybdenum on the Growing Chick.* (23219)

A. A. KURNICK,† B. L. REID, R. N. BURROUGHS, H. D. STELZNER AND J. R. COUCH
Departments of Poultry Science and Biochemistry and Nutrition, Texas A. and M. College System, College Station.

The role of molybdenum in animal nutrition has been ably reviewed by Dick(1) and the association of this element with xanthine oxidase in the rat by Westerfeld and Richert (2). The first indication of a possible requirement for molybdenum by the chick and poult fed a purified type diet was reported by Reid *et al.*(3). The addition of 0.0254 ppm of molybdenum to a purified diet has been subsequently shown by the same group of workers to stimulate the growth of poults and the activity of both intestinal and liver xanthine dehydrogenase(4). Furthermore, a portion of the growth promoting activity of the ash of distillers dried solubles(5,6) was reported by Reid *et al.*(4) to be due to the molybdenum content of this unidentified nutrient source.

The present study was initiated to determine the effect of feeding distillers dried solubles ash, after the molybdenum has been removed from this substance, on the growth, bone ash, molybdenum distribution in tissues and on xanthine dehydrogenase activity of chicks.

Method. New Hampshire chicks, from dams fed an all vegetable protein diet containing no added unidentified growth factors (5), were distributed at random into groups of 12 birds each. Each treatment was replicated once. The basal diet (Extracted soybean protein and Cerelose supplemented to meet the requirements of the chick) and the care and management of the birds was the same as previously reported by Reid *et al.*(3). At the close of the experiment, the chicks were sacrificed; the liver and a portion of the

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† Predoctoral Research Fellow of National Cancer Institute, N.I.H., P.H.S., Bethesda, Md.

TABLE I. Effect of Ash of Distillers Dried Solubles, after Removal of Molybdenum, on Growth, Bone Ash and on Molybdenum Distribution in Tissues of Chicks Fed a Purified Diet.

Supplements to basal diet	Mo content of diets (ppm)	Avg wt at 3 wk (g)	Response over basal (%)	Bone ash (%)	Mo content of bone ash (ppm)	Mo content of whole blood (γ/ml)	Mo content of liver, dry wt (ppm)
None	1.16§	228.3 (24)¶		51.2 (15)¶	.756**	.032**	1.84
3% distillers dried solubles	1.21	271.1 (24)	18.8‡‡	50.9 (15)	.605	.026	2.08
Distillers dried solubles ash*	1.21	249.0 (22)	9.1	52.3 (15)	.658	.031	2.81‡‡
*Idem,** (molybdenum removed)	1.16‖	225.9 (24)	−1.1	52.7 (15)	.775	.028	1.32
Sodium molybdate, .03 ppm Mo†	1.19	258.5 (24)	13.2††	51.8 (15)	.570	.038	2.17
" " , .3 ppm Mo†	1.46	258.6 (23)	17.8‡‡	50.3 (15)	3.210	.062	6.24‡‡
U. G. F. mixture‡	1.51	268.9 (22)	13.2††	51.1 (15)	.706	.042	3.11‡‡
L. S. D., .05		27.1	11.9				.64
" , .01		35.8	15.7				.87

* Supplemented in amt equivalent to 3% distillers dried solubles. † Supplied as $Na_2MoO_4 \cdot 2H_2O$. ‡ 6% distillers dried solubles, 2% antibiotic fermentation residue, 3% dried whey, 3% fish solubles and 3% forage juice. § Molybdenum content of basal diet based on 6 replicate analyses. ‖ Contained 0.004 ppm added molybdenum (not extracted). ¶ Figures in parentheses indicate No. of chicks or samples on which avg figures were based. ** Determination carried out on pooled samples within groups. †† Significant to the 0.05 level of probability over unsupplemented group. ‡‡ Significant to the 0.01 level of probability over unsupplemented group.

jejunum were removed for xanthine dehydrogenase determinations according to the procedure of Remy *et al.*(7). Homogenate nitrogen was determined by microKjeldahl according to the method recommended by A.O.A.C. (8). Blood was collected by heart puncture prior to sacrificing the birds. The left tibia from each sacrificed bird was severed and retained for bone ash and molybdenum determinations. All samples for molybdenum determination were digested with conc. HNO_3, H_2SO_4 and 70% $HClO_4$ according to the method described by Piper(9) and molybdenum content of the digest determined according to the method of Purvis and Peterson(10) using ethyl ether as extraction solvent. Molybdenum was removed from the ash of distillers dried solubles by digestion with HNO_3 and $HClO_4$, followed by ether extraction of the NH_4SCN-molybdenum complex produced in the digest with NH_4SCN and $SnCl_2$. Extraction of the complex was continued until after the concentration of molybdenum in the ether extract was determined colorimetrically and found to correspond to the molybdenum concentration previously found in 5 replicate samples (18.74 ppm). The residue was evaporated to dryness, taken up in redistilled water and neutralized with NH_4OH. All supplements were added at the expense of glucose.‡ The duration of the experiment was three weeks.

Results. Addition of either distillers dried solubles, sodium molybdate (0.03 or 0.3 ppm Mo) or a mixture of 5 unidentified nutrient sources to the basal diet resulted in a significant growth increase compared to that observed in the control group (Table I). The growth response due to the ash of distillers dried solubles (9.1%) was one-half that observed with distillers dried solubles (18.8%). These data are in agreement with previous reports(5,6). However, the response of the group fed this ash sample was not sufficiently great to be statistically significant. Removal of molybdenum from the ash resulted in a 1.1% growth depression as compared to the basal group.

With the exception of the ash of distillers

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‡ Cerelose.®

TABLE II. Effect of Ash of Distillers Dried Solubles, after Removal of Molybdenum, on Intestine and Liver Xanthine Dehydrogenase Activity of Chicks as Compared to Other Sources of Dietary Molybdenum.

		Xanthine dehydrogenase activity,¶ cmmo$_2$/20 min.		
			Liver	
Supplement to basal diet	Mo content of diet (ppm)	Intestine, per 283 mg (wet wt)	per 283 mg (wet wt)	per mg of N
None	1.16§	22.5	100.4	7.95
3% distillers dried solubles	1.21	34.4‡‡	120.2‡‡	8.92††
Distillers dried solubles ash*	1.21	30.7††	101.4	8.11
Idem,* (molybdenum removed)	1.16‖	25.0	96.9	7.53
Sodium molybdate, 0.03 ppm Mo†	1.19	31.8††	128.4‡‡	10.02‡‡
" " , 0.3 ppm Mo†	1.46	37.0‡‡	137.4‡‡	10.53‡‡
U. G. F. mixture‡	1.51	32.3††	137.5‡‡	10.01‡‡
L. S. D., 0.05		8.0	11.6	.89
" , 0.01		10.6	15.4	1.18

*–‖ See Table I.

¶ A 1:6 dilution of tissue homogenate was used in these studies.

dried solubles, all supplements which supplied at least 0.03 ppm of molybdenum above that found in the basal diet exerted a significant growth response. This was not unexpected in view of the previously reported growth increases when only 0.0126 ppm of molybdenum as molybdic acid was added to the basal diet(3). These data would indicate that the chick can utilize higher levels of molybdenum for optimum growth. However, addition of higher molybdenum levels (0.3 and 0.35 ppm) from either organic or inorganic sources, did not result in increased growth above that observed with the lower supplemented levels (0.03-0.05 ppm).

Slight differences in bone ash values found in the groups fed the supplements were not statistically significant (Table I). However, further investigations are warranted in view of the work of Morrison *et al.*(11) in which elevated bone ash values resulted in birds fed a purified diet supplemented with the ash of a mixture of unidentified factor sources.

Molybdenum contents of the tissues studied indicate that low levels of molybdenum supplied either by distillers dried solubles, the intact ash of the supplement or by sodium molybdate did not significantly affect the molybdenum concentration of bone ash or whole blood; but did affect the molybdenum concentration of liver (Table I). The addition of 0.3 ppm of molybdenum as sodium molybdate to the basal diet increased molybdenum deposition in the tissues studied. Since bone ash and blood samples were pooled for the molybdenum determinations, the differences found are not considered to be significant.

The livers of chicks fed the molybdenum free ash exhibited the lowest liver molybdenum concentration; whereas, the group fed the untreated ash showed a significant ($P < 0.01$) increase in molybdenum deposition over the basal group (Table I). The ash of distillers dried solubles supplied 0.05 ppm of molybdenum to the diet which was equal to that supplied by the unashed material; however, untreated distillers dried solubles failed to affect the level of molybdenum in liver. Livers of chicks fed 0.3 ppm of molybdenum as sodium molybdate showed the highest concentration of the element ($P < 0.01$). The liver molybdenum deposition in this group was significantly higher than that observed in the group fed the mixture of unidentified growth factors which supplied 0.35 ppm of molybdenum.

Liver and intestine xanthine dehydrogenase activity were significantly increased by the added molybdenum sources (Table II). The treated ash (molybdenum removed) failed to increase the enzyme activity either in intestine or liver above that observed in the basal group. The enzyme activity of the group fed the intact ash exhibited a significant response ($P < 0.05$, Table II), in intestinal xanthine dehydrogenase, but failed to increase the liver enzyme activity. Rat intestine was found by Westerfeld and Richert(2) to be a better test object than liver tissue for the unidentified liver residue factor. This may help to explain the difference in response observed with the 2 tissues on the same treatment.

No significant differences between intestinal enzyme activity of the dietary treatments other than the control group were noted. Since the supplements supplied varying levels of molybdenum (0.05-0.35 ppm), it was assumed that a saturation level has been reached similar to that observed in the rat(12).

The response of liver xanthine dehydrogenase activity to the added supplements was less consistent than that observed in intestinal homogenates. The liver enzyme activities of the groups fed the higher level of sodium molybdate (0.30 ppm) and the U.G.F. mixture (added Mo 0.35 ppm) were significantly higher ($P<0.01$) than the activity noted in the livers of birds fed distillers dried solubles supplying 0.05 ppm of molybdenum. When xanthine dehydrogenase activity was expressed on a nitrogen basis the differences observed above were quantitatively less, but still statistically significant ($P<0.05$).

The ratio of enzyme activity to liver molybdenum content was more erratic than that reported in turkey poults(4). However, a correlation of 0.617 ($P<0.05$) was found to exist between the results of the two determinations.

Summary. 1) Growth of chicks was significantly improved by the addition of distillers dried solubles, sodium molybdate or a mixture of unidentified factor sources. Removal of the molybdenum from the ash of distillers dried solubles resulted in the same growth rate exhibited by the control group. The ash of this product produced a 9.1% increase in growth; which was not quite significant. 2) Bone ash values were not affected by the dietary treatments. Intestinal xanthine dehydrogenase activities were significantly increased in all cases except the "molybdenum-free" distillers dried solubles ash-fed group.

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Cold-Pressor Responses in Families of Rabbits with Spontaneous Hypertension.* (23220)

NATALIE ALEXANDER, WILLIAM J. TIBBS[†] AND DOUGLAS R. DRURY
Department of Physiology, School of Medicine, University of Southern California, Los Angeles.

For the past 5 years we have bred rabbits with spontaneous hypertension(1). A high percentage of their progeny inherit the hypertension(2). We recently began raising families of control rabbits under the same conditions as those of the spontaneously hy-

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[†] In part derived from thesis to Univ. of S. California Graduate School in partial fulfillment of requirements for the M.S. in Physiology.

pertensive group. Controls are offspring of rabbits chosen at random from normotensive laboratory stock. They are from the same strains that show spontaneous hypertension—New Zealand and Dutch.

We set out to see whether spontaneously hypertensive rabbit families differed from normotensive laboratory stock in their blood pressure response to cold stimulation. Hines and Brown developed a cold-pressor test in humans to evaluate what they term vascular reactivity(3), and we adapted this test for rabbits in the present study. Hines and Brown showed that persons with hypertension or normotensives with a family history of hypertension tend to have a greater blood pressure response to the cold-pressor test than persons with no personal or family history of hypertension(4). Progeny of the spontaneously hypertensive rabbits used in this study will be referred to as "selected" animals and controls as "unselected" animals.

Methods. Cold-pressor test. Results from cold-pressor tests are meaningful only if the conditions, *i.e.,* stimulus and environment, of the tests are identical(3,5). Our procedures were standardized and all conditions rigidly controlled. Further, to insure that the response would be solely to the stimulus applied, the animals were sedated with pentobarbital (Nembutal—Abbot Vet) 15 mg/kg. *Cuff method.* The abdominal cuff method of McGregor(6) is used routinely to determine arterial blood pressures in our colony of rabbits (2) and was used in the first series of cold-pressor tests. Experience with the cuff method has shown that diastolic pressure probably cannot be determined with an accuracy greater than ±10 mm Hg, but systolic pressure may be obtained with an accuracy of ±4 mm Hg. Therefore, only systolic pressures are reported from these tests. After a set of control blood pressure readings was obtained, the sedating dose of pentobarbital was injected into the marginal ear vein. Blood pressure fell immediately, then gradually rose and stabilized within 27 to 45 minutes. This stable pressure under sedation will subsequently be referred to as the *basal* pressure. At this time animals were relaxed, had normal pupils and a corneal reflex. If handled gently the sedated animals offered no resistance, and ordinary laboratory sounds did not evoke any response. When the basal condition had been achieved, the ears were completely immersed in a beaker of ice water (2° to 4°C) for exactly 60 seconds. Systolic and diastolic pressures were determined every 10 to 15 seconds during the "cold minute" and for 2 minutes after the ice water was removed. *Intra-arterial method.* In a second, smaller series of cold-pressor tests, femoral arterial pressure was recorded with a #24 needle connected to a Statham P-23A pressure transducer. Oscillographic records were made at a film speed of 0.4 inch/second. The femoral artery was exposed after subcutaneous infusion of 1% procaine; the recording needle was inserted 30 to 35 minutes after Nembutal injection. Pressure recorded 10 to 30 seconds *before* immersing the ears in ice water was considered the basal pressure (see above). The needle remained in the artery throughout the "cold minute" and for 2 minutes afterwards. Both systolic and diastolic pressures were measured to within ±2 or 3 mm Hg, and both pressures are reported. *Calculation of response to cold-pressor test.* The value for maximum systolic or diastolic pressure response obtained from an intra-arterial record was the difference between the highest pulse (or pulses) of the basal pressure and highest one(s) recorded during the "cold minute." The time at which the highest pressure first occurred during the "cold minute" was not necessarily the same for systolic and diastolic pressure. Maximum response was obtained *during* the "cold minute" in 35 of 40 tests by the intra-arterial method. Since only 5 maximum responses occurred after the "cold minute" (longest time was 115 seconds from time of ear immersion), we felt justified in disregarding these and based all results on the maximum response during the "cold minute." Since pressure determinations by the cuff method were intermittent (every 10 to 15 seconds), maximum responses obtained beyond the "cold minute" were included in the results. (The longest time to a maximum response was 103 seconds after immersion of ears.)

Results. The cuff method was used on 56

TABLE I. Maximum Systolic and Diastolic Responses to Cold-Pressor Test, Recorded by Intra-Arterial or Abdominal Cuff Method, in Unselected and Selected Rabbits.

	No. trials	Max. systolic response Mean (mm Hg) and S.D.	Max. systolic response Avg time to max. resp. (sec.)	Max. diastolic response Mean (mm Hg) and S.D.	Max. diastolic response Avg time to max. resp. (sec.)
Intra-arterial method					
Unselected	23	8.4 ± 4.1	43	7.0 ± 4.7	44
Selected	17	16.5 ± 8.9	39	13.4 ± 6.6	38
Cuff method					
Unselected	26	8.7 ± 4.9	40		
Selected	108	14.8 ± 6.2	38		

Calculation of Fisher's ''t'' values showed that the means of maximum systolic responses for unselected and selected rabbits by both abdominal cuff and intra-arterial methods are ''very significantly different'' ($p = <.001$), and the means of maximum diastolic response by intra-arterial method are ''significantly different'' ($p = <.01$).

selected and 15 unselected rabbits. Two tests were carried out on most animals 1 to 6 days apart, and the results compiled from all the determinations. If an animal struggled during the test, the results were discarded. A total of 108 tests on selected, and 26 on unselected, were successful. For the intra-arterial method, 15 selected and 15 unselected rabbits were used. Most of the unselected animals were tested 2 times and some 3, but only 23 tests were considered satisfactory. The others were discarded because the recorded pulse wave was damped. Only 2 repeats were successful in the selected group giving a total of 17 tests. Some of the animals were used for both cuff and intra-arterial tests.

The averages and p-values for maximum pressure responses and time (seconds) to maximum response are given in Table I for both intra-arterial and cuff tests. Selected rabbits had "significantly" higher mean maximum pressure responses than unselected rabbits in both series of tests. Although values overlapped, some selected animals had maximum responses above the highest ones in the unselected group.

Table II shows that selected animals had a greater average maximum systolic response than unselected rabbits at any given basal pressure range. Additionally, it can be seen that there was no correlation between maximum systolic response and the basal systolic pressure for either group. The same was true in repeat tests on single animals. For example, a cold-pressor test was repeated by the cuff method 3 times at 5 minute intervals in an unselected rabbit. The systolic responses were 6, 4, and 5 mm Hg with basal pressures of 130, 123, and 116 mm Hg, respectively.

In a group of 5 rabbits, 2 or 3 cold-pressor tests were repeated at 5 to 15 minute intervals. The magnitude of response to each test by a single animal was similar, not differing by more than 6, and usually by only 1 or 2, mm Hg. On the other hand, the magnitude of response to tests made from 1 to 6 days apart was not necessarily the same in a given animal, differing by as much as 30 mm Hg.

The cold-pressor responses of 5 selected

TABLE II. Average Maximum Systolic Pressure Response of Selected and Unselected Rabbits with the Same Basal Systolic Pressures—Intra-Arterial Method.

	Basal systolic pressure* (mm Hg) 91–100	101–110	111–120	121–130	131–140	151–160
Unselected	10 (1)†	7 (5)	8.5 (6)	7.5 (6)	9.5 (5)	
Selected	24 (1)	13 (3)	16 (7)	16 (3)	15 (2)	29 (1)

* Avg basal pressure for selected and unselected groups was 120 and 119 mm Hg, respectively.

† No. of animals.

animals with consistently high[‡] systolic pressures were compared to those of 5 selected animals with consistenly low[§] systolic pressures. There was no apparent relationship between these levels of blood pressure and the magnitude of response to the test.

Discussion. We previously pointed out certain cardiovascular characteristics of spontaneously hypertensive rabbits similar to those found in human essential hypertension (2). The most obvious similarities are elevation and lability of systolic and diastolic pressures. In the present study another cardiovascular characteristic of human essential hypertension was demonstrated by progeny of rabbits with spontaneous hypertension, *viz.*, as a group they showed a "significantly greater" average blood pressure response to the cold-pressor test than rabbits from normotensive families.

The intra-arterial records showed that maximum variation in heart rate during the cold-pressor test was only 5% of the basal rate. Thus, the mechanism for large pressure responses in selected rabbits was probably not attributable to changes in cardiac output.

The selected animals sometimes responded with pressure increases 2 to 3 times those of the unselected group. This difference in responsiveness to the cold-pressor test was not correlated with a higher starting or basal level of pressure (Table II) in selected animals, but rather it seems to be a characteristic of the animal. Further evidence that an increased cold-pressor response in selected animals is a characteristic of these rabbits and not dependent on the presence of elevated pressure is that those with consistently normal pressures showed greater blood pressure increases than did unselected normotensive rabbits.

Summary. (1) Blood pressure responses to cold-pressor test were studied in progeny of spontaneously hypertensive rabbits. These animals were both hypertensive and normotensive. Controls were offspring of rabbits chosen from normotensive laboratory stock. (2) Average maximum blood pressure response to cold stimulation of progeny of spontaneously hypertensive rabbits was quantitatively greater than in the control group. This difference in responsiveness was not attributable to difference in basal (starting) pressure. (3) It is concluded that increased pressure response to cold is a characteristic of progeny of spontaneously hypertensive rabbits.

[‡] > 150 mm Hg
[§] < 130 mm Hg
as determined by routine cuff measurements 2 or 3 times a month from age 3 or 4 months. Ages of animals at time of tests ranged from 7 to 10 months.

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Effect of Small Measured Doses of Radiation on Lymphocyte Morphology in Man. (23221)

R. Lowry Dobson and Mary M. Chupp (Introduced by J. H. Lawrence)
Donner Laboratory of Medical Physics and Biophysics, and Radiation Laboratory, University of California, Berkeley.

Ingram and Barnes(1) reported an increased incidence of bilobed and binucleated lymphocytes in the circulating blood of cyclotron workers. When these observations came to our attention a program was initiated at this laboratory to investigate the phenomenon on a quantitative basis. A study was carried out that same year on 5 groups of laboratory personnel comprising 34 persons(2). The results suggested an increase in incidence of binucleated lymphocytes in individuals exposed intermittently or chronically to radiation at levels below 300 mr per week. Animals given doses of x- and γ-rays from 2 to 75 r have also shown this response(3,4,5). Ingram *et al.*(6) presented additional data on the original group of cyclotron workers, and demonstrated a decrease in abnormal lymphocytes following shielding of the accelerator.

Presented here are results of a study of the binucleated lymphocyte response in normal individuals chronically exposed to low doses of ionizing radiation, predominantly x- and γ-rays.

Methods. The incidence of binucleated lymphocytes in the peripheral blood of 8 accelerator operators was determined within a few hours to a few days after exposures of 80 to 200 mr. Comparison was made with the incidence in a closely matched control group of 7 persons receiving no known radiation exposure above background levels. Intermittent and chronic exposures to x- and γ-rays were measured with electroscopes and film badges. These values were checked with independent measurements of radiation fields in work areas. The average exposure level of the accelerator operators during the year prior to sampling was 110 mr per week. During the week prior to and including the taking of blood samples the average for the group was 190 mr. The neutron fields to which these persons were exposed were low in comparison. In the one instance where neutrons could be considered to have contributed significantly to the total exposure the individual received on the average only 13% of the maximum permissible weekly neutron dose. The other individuals in the group received considerably lower neutron exposures, of the order of 3% to 6% of the maximum permissible weekly exposure. Measurements were made by comparing track counts in the emulsion of neutron film badges to emulsions exposed to a standard Po-Be source. The maximum permissible weekly dose was taken as 4.3×10^6 Po-Be neutrons per square centimeter. Using a relative biological effectiveness of 10 this represents a dose of 300 mrem per week.

Results. The lymphocyte data presented in Fig. 1 represent the counting of more than one million white blood cells. An average of 58,000 cells per person was examined in the exposed group, an average of 77,000 cells per person in the control group. Counts were made from stained blood smears. At least 5 individual counts were taken from each person at various times of day and totaled. Only binucleated lymphocytes with 2 completely separate nuclei or with 2 non-overlapping nuclei connected by a thin hair-like filament were recorded. Cells not meeting these criteria and lymphocytes with bilobed nuclei were not considered in this study. In order to avoid possible artifactual changes, concentration methods and anticoagulants were not used.

It is apparent that the incidence of binucleated lymphocytes is low, and that more of these cells were seen in the exposed individuals than in the controls. The mean in the exposed group was 5.9 per 50,000 white blood cells with a range of 3.4 to 7.6; it was 1.4 per 50,000 in the control group with a range of 0.4 to 2.5. The difference is highly significant by statistical tests.

In an attempt to explore the pattern of this lymphocyte response to individual and cumulative exposures, several persons were studied

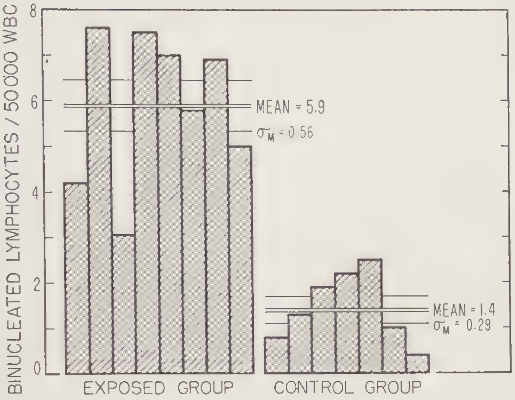

FIG. 1. Incidence of binucleated lymphocytes in peripheral blood of 7 normal unexposed individuals and of 8 normal persons exposed to levels of ionizing radiation of the order of 200 mr per week.

over periods of many months. On one of these persons 17 counts, each consisting of at least 5 hourly samples were taken during a 90-day period shortly after he began working with radiation. These data from the counting of 936,000 white blood cells are of interest because his average radiation exposure was 239 mr per week; and during this period he received 4 individual daily exposures exceeding 200 mr. While the first 2 counts showed only 1.5 and 0 binucleated lymphocytes per 50,000 white cells respectively, the incidence during the rest of the interval fluctuated about a mean of 6 per 50,000. In general larger numbers were seen several days after exposures to 200 mr or more. Daily counts were taken frequently during a 10-day period following a single dose of 264 mr on the eightieth day. Forty-eight hours after this exposure the incidence of binucleated lymphocytes was 15 per 50,000 white blood cells; 10 days after exposure it was 17 per 50,000; the average daily count between these 2 times was 3.5 per 50,000. It does not appear possible at this time to adequately relate a pattern of the binucleated lymphocyte response to individual radiation doses; however, such a relationship is suggested by these observations and is currently under study.

Discussion. Binucleated lymphocytes occur in small numbers in the peripheral blood of normal individuals. High numbers have been seen in patients with lymphatic leukemia; they may be elevated in hepatitis, and probably in other abnormal conditions. The data reported here were obtained from persons in good health. Physical findings, laboratory data and medical histories were reviewed in each case. It must nevertheless be borne in mind that this response is not specific to ionizing radiation. Murphy and Sturm(7) described seeing large numbers of these cells in the blood of animals after heat treatments. The possible relationship of these cells to the "stress lymphocytes" of Frank and Dougherty(8) is being investigated.

Summary. In the study reported here a significant increase in incidence of binucleated lymphocytes is demonstrated in persons exposed to ionizing radiation at levels of the order of 200 mr per week.

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Metabolism of l-Thyroxine and l-3:5:3′-Triiodothyronine by Homogenates of Rat Skeletal Muscle.* (23222)

JAMSHED R. TATA[†] (Introduced by C. J. Shellabarger)

Division of Clinical Investigation, Sloan-Kettering Institute for Cancer Research, New York City.

It has been shown recently(1) that tissue slices and homogenates of rat brain can deiodinate and possibly deaminate l-thyroxine and l-3:5:3′-triiodothyronine. Comparative studies using rat liver and kidney proved technically difficult because incubation of thyroxine and triiodothyronine with either slices or homogenates of these 2 tissues produced between 4 and 7 different iodinated metabolites. Most of these products were not formed by brain preparations, in which the iodinated metabolites were mainly inorganic iodide and what appeared to be the deaminated analogues of thyroxine and triiodothyronine. Subsequently it was found that the metabolites produced from homogenates of muscle resembled, to some extent, those produced by brain homogenates. These observations are described in the present report.

Methods. About 2-3 g of skeletal muscle were removed from each hind leg of unanaesthetized, decapitated male or female rats (75-150 g). Muscle was rapidly chilled in ice, trimmed and cut into fragments of approximately 100 mg. The tissue was then homogenized in a Waring blendor run at 65-75 V for 3 minutes.[‡] For incubation studies a concentration of 1 g of muscle per 15 ml of suspension medium was used. Radioactive (I^{131}) l-thyroxine[§] and l-3:5:3′-triiodothyronine[§] were used for incubation with homogenates. Incubation time ranged from 15 minutes to 6 hours. Substrate levels between 10^{-3}M and 2 x 10^{-9}M were obtained by addition of non-radioactive thyroxine and triiodothyronine when necessary. After incubation each sample was chromatographed directly on paper to determine the amount of I^{131} remaining as unchanged substrate, as well as the amount of each of the metabolites. Comparative studies were simultaneously done on brain homogenates from the same animals. Analytical procedures, the composition of the suspension medium, conditions of incubation and control experiments were the same as those previously reported for rat cerebral tissue(1).

Results. Since anaerobic conditions failed to show any significant difference, only results of incubation under aerobic conditions are reported. The rates of disappearance of thyroxine and triiodothyronine at different substrate levels are shown in Fig. 1. The data here have been expressed as percentage of the total homogenate radioiodine remaining after incubation as either thyroxine or triiodothyronine. Simultaneous studies with brain homogenates were similar to those reported previously(1) and are not repeated here.

The disappearance of radioactive thyroxine and triiodothyronine from muscle homogenates was most rapid during the first 2 hours of incubation. At roughly the same substrate levels rates of disappearance were substantially faster with muscle homogenates than those obtained with homogenates of brain per g of tissue. For example, brain suspension was almost inactive on thyroxine and triiodothyronine at a concentration of about 10^{-6} M, while similar muscle suspension (1 g tissue/15 ml medium) metabolized approximately 60% of thyroxine and 35% of the triiodothyronine within 3 hours of incubation. This difference might indicate a higher concentration, in muscle, of the enzyme(s) involved in the metabolism of the 2 hormones. In both muscle and brain homogenates thyroxine was degraded more rapidly than tri-

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[†] Present address Nat. Inst. for Medical Research, Mill Hill, London, N.W.7, England.

[‡] Most of the connective tissue which collects around the blades of the blendor was removed after one min. Homogenization was then continued for an additional 2 min.

[§] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

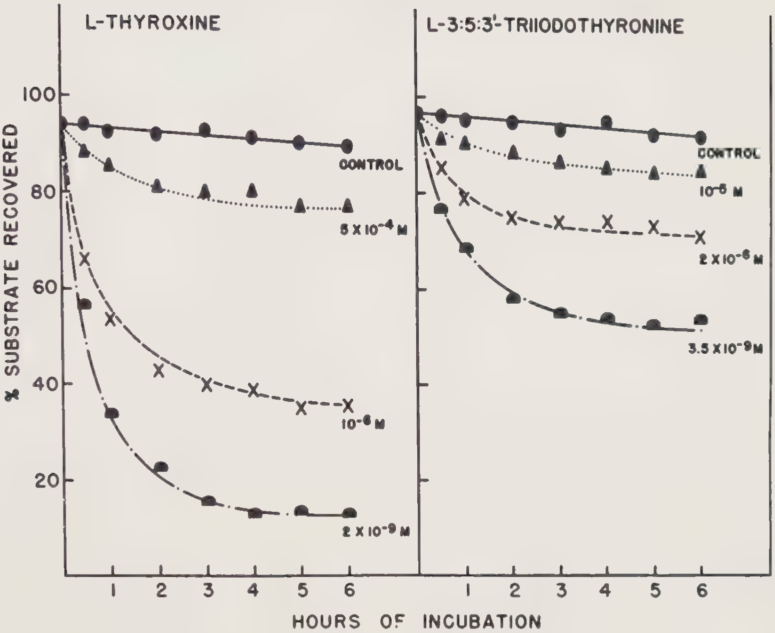

FIG. 1. Rates of degradation of I^{131}-labelled l-thyroxine and l-3:5:3′-triiodothyronine incubated with rat muscle homogenate at different substrate concentrations. Values given represent percentage of total I^{131} in homogenates found as unchanged substrate at different time intervals. c = control incubation with boiled tissue.

iodothyronine. An explanation for this difference is not apparent.

Chromatographic analysis of the iodinated products obtained after incubation of labelled thyroxine and triiodothyronine with muscle and brain homogenates is shown in Table I.

The major I^{131}-labelled metabolite in all cases‖ was inorganic iodide. In the case of muscle homogenates, inorganic iodide appeared to be the main labelled metabolite from thyroxine or triiodothyronine since, in all studies, at least 90% of the I^{131} of the product formed was found in this fraction. As much as 10-15% of the total I^{131} was found as their acetic and/or propionic acid analogues (it was not possible to distinguish chromatographically between the acetic and propionic acid analogues of the 2 hormones) within 1-2 hours of incubation with brain homogenates. Using muscle homogenates, however, it was not possible to obtain sufficient radioactivity to elute and re-chromatograph any of the non-iodide fractions of the iodinated metabolites. 3:5:3′-Triiodothyroacetic acid, identified in muscle of thyroidectomized rats injected with L-T_3 by Roche and co-workers(2) was not found to be present as a metabolite of incubation in this work.

Both muscle and brain homogenates were inactivated by heating at 100°C for 20 minutes. Treatment of brain preparations with 10^{-3} M $HgCl_2$ blocked all deiodination but did not interfere with conversion of thyroxine and triiodothyronine to other non-deiodinated products. In muscle preparation 10^{-3} M $HgCl_2$ blocked all deiodination but, as may be seen in Table I, since the non-deiodinated products represented such a small fraction of the total I^{131} it was difficult to be certain of any effect on conversion of thyroxine and triiodothyronine to other products.

The process of homogenization of the tissue is not responsible for deiodination or lack of deamination of l-thyroxine and l-3:5:3′-triiodothyronine since no significant qualitative difference was obtained by incubation with skeletal muscle fragments.

‖ In considering the qualitative analysis of radioiodinated metabolites, it should be emphasized that the iodine atoms in molecules of thyroxine and triiodothyronine used for this work were not uniformly labelled.

TABLE I. Breakdown of I^{131} Labelled l-Thyroxine (T_4) and l-3:5:3′-Triiodothyronine (T_3) by Rat Brain and Muscle Homogenates (1 g Tissue/15 ml Medium).

		l-Thyroxine, 2×10^{-9}M				l-3:5:3′-Triiodothyronine, 3.5×10^{-9}M			
		% total I^{131} distributed as*				% total I^{131} distributed as*			
Tissue	Hr incubation	T_4	I^-	Or.	Others†	T_3	I^-	Or.	Others†
Muscle	.5	57.1	40.4	1.3	+	76.6	22.7	+	1.4
	1	34.5	63.5		2.0	69.0	29.0	2.0	
	3	17.3	79.1	3.6		56.6	32.4		1.0
	6	12.6	84.2	2.5	+	53.0	35.5	1.5	
Boiled muscle	6	86.1	12.0	+	+	92.4	6.8	+	
Brain	1	68.7	15.9	+	14.8	80.1	9.2	+	10.3
	3	60.4	27.0	3.8	8.8	69.3	23.4	2.7	4.6
	6	51.0	33.2	3.8	2.0	64.8	34.2	1.0	
Boiled brain	6	89.8	10.2			90.6	8.3	+	

* + = Less than 1%; Or. = Fraction of I^{131} immobile in chromatography.

† In case of the brain this group includes acetic acid and/or propionic acid analogues of T_4 and T_3.

Summary. From the present study it can be concluded that brain and muscle have certain similarities as well as differences in the metabolism of thyroxine and triiodothyronine. The deiodinating enzyme is present in both the tissues but the deaminating activity found in brain preparations is virtually absent in skeletal muscle. The nature of the enzymes as well as other factors involved in the metabolism of these hormones require further clarification and are being studied.

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Inhibitory Effects of Cortisone Acetate and Hydrocortisone on Growth of Fibroblasts.* (23223)

MARGARET HOLDEN AND LILLIAN B. ADAMS (Introduced by H. M. Rose)
Department of Microbiology, College of Physicians and Surgeons, Columbia University, N. Y. City.

Diverse evidence has been presented by several investigators concerning the direct action of cortisone acetate and 17-hydroxycorticosterone on growth of fibroblasts *in vitro*. The technics have differed as to methods of culture and application of the test agents(1-7).

The purpose of the present work was to study the effect of cortisone and hydrocortisone on a single cell system. The results indicate that addition of these steroids to cultures of actively proliferating fibroblastic cells inhibits their growth and induces development of abnormal cytologic changes.

Materials and methods. The steroids, cortisone acetate and hydrocortisone (free alcohol) were supplied through the courtesy of Dr. Frederick Heath, Merck & Co. Cortisone was diluted in aqueous vehicle to contain 5 mg/ml and further diluted to desired strength with Earle's balanced salt solution (8) prior to combining with the culture media. Hydrocortisone was prepared according to the method of Grossfeld and Ragan(3). The original vehicle was removed from this steroid and replaced by saline. To 1 ml of this suspension were added 1.25 ml of 95% ethyl alcohol and 0.75 ml of distilled water. A clear solution containing 8.33 mg/ml of hydrocortisone thus obtained was further diluted with saline to proper concentration for use in the tests. Cholesterol suspended in saline was employed as a control in several experiments. Stock cultures of fibroblasts (L strain, Earle)(9) were grown on the surface of T-60 flasks in a nutrient mixture of horse serum (40%), embryo extract (20%), and Earle's saline (40%). After 7 to 10 days of cultivation the cells were loosened from the glass surface by gentle shaking, suspended in 0.025% trypsin solution at a pH 7.4 to 7.6, incubated at 37°C for 20 to 30 minutes to break cellular clumps and occasionally agitated to obtain an uniform suspension of single cells. Following centrifugation at 1000 rpm for 10 minutes and removal of the supernatant trypsin solution, the cells were resuspended in nutrient medium alone or in medium containing 10 μg/ml of cholesterol, cortisone or hydrocortisone. The cellular concentration was measured by direct count in a hemocytometer. In earlier studies the cells were diluted in balanced salt solution containing neutral red but the vital dye was later discontinued, since healthy-appearing cells were readily discernible without staining. Two and five-tenths milliliter aliquots of the suspensions were dispensed by an automatic syringe into wide mouth, flint glass, screw top bottles. Four bottles were set up for each combination of cells and reagents and incubated at 37°C. During the test period the nutrient medium, with or without steroids, was replaced every 2 or 3 days to maintain the cells. After 11 days the cells from the bottles of each combination were pooled, treated with trypsin, centrifuged, resuspended in saline and enumerated as before. Growth rates were studied in cultures containing 10, 20, and 50 μg/ml of hydrocortisone. In these experiments the treated and untreated cells from 2 bottles of each combination were pooled and counted at 3- or 4-day intervals for 14 days.

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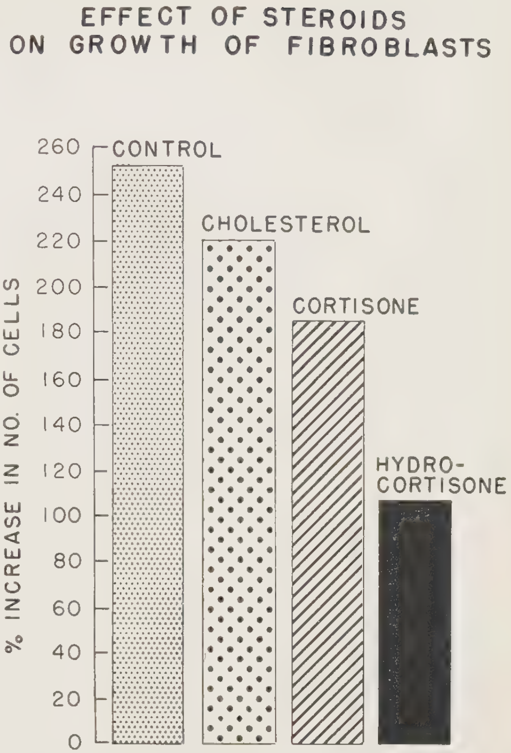
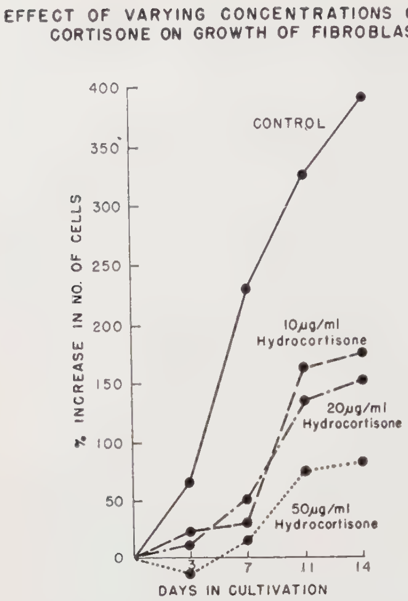
* This investigation was supported by research grant of the N.I.H., Public Health Service.



GRAPH I. Retarding effect of 10 μg/ml of cholesterol, cortisone or hydrocortisone on mouse fibroblasts grown for 11 days. The data from 9 experiments have been pooled.

Cultures of fibroblasts derived from subcutaneous areolar tissue of adult rats were used in a series of experiments designed to test effect of cortisone acetate and hydrocortisone on cell mitosis. The cultures were carried for 15 days or longer in roller tubes and then transferred to Maximow slides(10). After 5 days, when they were about to reach their maximal growth rate, they were exposed to 20 μg/ml of the steroids in the feeding solution. In control cultures saline was substituted for the test agents. In these experiments the feeding solution was composed of human placental serum (20%), beef serum ultrafiltrate (20%), embryo extract (20%), and saline (40%). At 4, 8, 12, 16, 20, and 44 hours, 2 to 4 slides containing 6 to 12 explants were fixed in methyl alcohol and stained with Jenner Giemsa. An average of 2000 cells for each time interval and for each agent were counted, using a Howard ocular grating. The percentage of these cells in the 4 phases of mitosis was determined.

Results. The data in Graph 1, compiled



GRAPH II. Curves illustrate retarding effect of 10, 20 or 50 μg/ml of hydrocortisone on mouse fibroblasts over a 14-day period. The data from 4 experiments have been pooled.

from 9 experiments, show the average percentage increase in number of cells over the original cell count in the control and steroid-treated cultures. The counts were made after 11 days. In control cultures there was a 253% increase in number of cells over the initial inoculum, whereas in cultures containing 10 μg/ml of cholesterol, cortisone or hydrocortisone, the percentage increase in number of cells was 220%, 185%, and 106%, respectively.

Having obtained evidence that hydrocortisone produced more inhibition than cortisone acetate, the next experiments were carried out using only hydrocortisone. Three concentrations of hydrocortisone were studied. The percentage increase of cell count over the original count at intervals of 3 or 4 days is shown in Graph II, which represents the average results of 4 experiments. This graph indicates that all 3 concentrations of hydrocortisone, 10, 20, and 50 μg/ml, produce a definite inhibiting effect on growth of fibroblasts. The inhibitory effect is most noticeable within the first 7 days. Both 10 and 20 μg/ml of hydrocortisone produce approximately the same degree of inhibition of growth. It may be observed that cultures containing 10 or 20 μg/ml of hydrocortisone produce about 50% fewer cells when compared to cell growth in control cultures. Fifty micrograms per ml are roughly twice as inhibitory to growth as the lesser amounts of hydrocortisone.

To determine whether damage to the cells by the steroids was reversible, the treated cultures were transferred into medium without the addition of the test agents; complete recovery of the culture with growth equal to that of control cultures ensued.

Typical changes in morphology were readily seen in unstained as well as in stained fresh preparations. Fig. 1 illustrates a fresh preparation of a normal control 10-day-old culture. The typically fibroblastic cells with the characteristic spindle-shape or, less frequently, with triangular shapes and long terminal processes may be seen. An occasional giant cell is present. In contrast, hydrocortisone-treated cells, Fig. 2, are less spindle-shaped and show definite shortening of their terminal processes; the cells appear swollen, transparent, flattened, and tend to cohere in

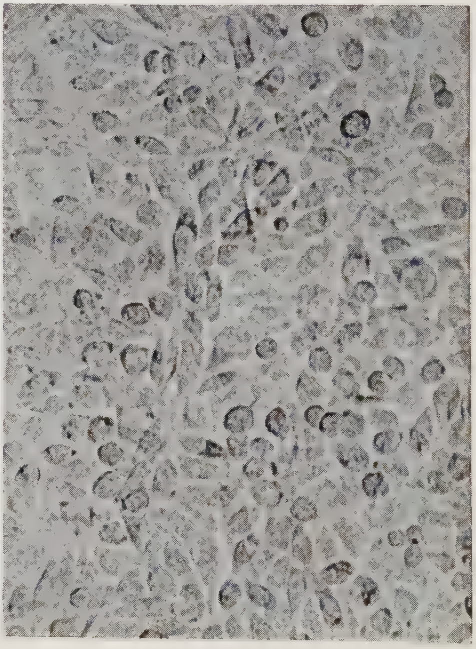


FIG. 1. Fresh preparation of normal control mouse fibroblasts (L strain, Earle) from a 10-day culture. Characteristic spindle-shaped cells and an occasional giant cell can be seen. X 100, approximately.

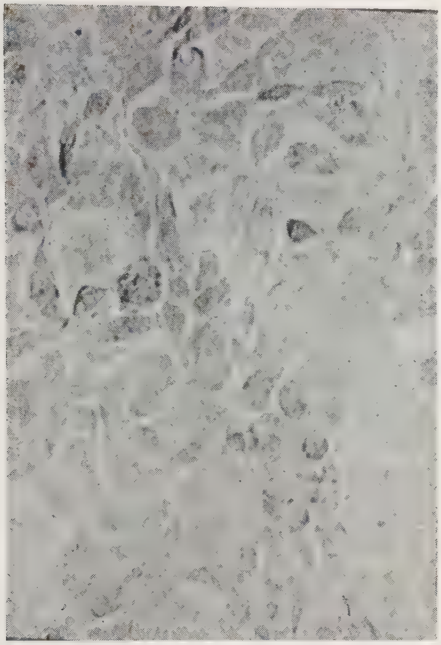


FIG. 2. Fresh preparation of mouse fibroblasts grown for 10 days with 50 μg/ml of hydrocortisone. In comparison to Fig. 1, the cells are swollen and less spindle-shaped. More giant cells are present. X 100, approximately.

sheets; some contain one single enormous nucleus, others 4 to 6 nuclei. Another constant effect of hydrocortisone on fibroblasts is the production of numerous cytoplasmic vacuoles (Fig. 3). These morphologic changes are present in cortisone acetate as well as in hydrocortisone-treated cells and become more pronounced with increasing concentrations of the steroids.

The reaction to the hormone was also noticeable following trypsinization. After 3 or more days' growth in the media containing steroids, detached normal cells, seen in the counting chamber, were round and uniform in size, unlike steroid-treated cells which were large, ameboid and irregular in outline.

Cortisone acetate and hydrocortisone in a concentration of 20 μg/ml did not arrest mitosis in any particular phase and there was no accumulation of one type of mitotic figure at any time during cultivation. In the 4 experiments carried out, there was no appreciable difference in mitotic index between the control and the steroid-treated cells. However, it should be noted that in these experiments the cells were rat fibroblasts and cultivated in a plasma clot.

Discussion. The experimental data indicate that hydrocortisone, and to a lesser degree cortisone acetate, inhibited growth of fibroblasts in tissue culture. Cholesterol caused minimal inhibition. The question arises whether the lesser effect of cortisone, as compared with hydrocortisone, might be due to the fact that cortisone (acetate) was in suspension and was inhomogeneously distributed in the medium, whereas hydrocortisone was in solution. Grossfeld and Ragan (3) tested these steroids in hanging drop chick embryo cultures and found that inhibition by hydrocortisone in suspension was greater than by cortisone acetate in suspension. They believed this indicated that factors other than water solubility make hydrocortisone more effective than cortisone acetate.

The finding that the inhibitory effect on growth of fibroblasts was greater when the concentration of steroids was increased from 10 or 20 to 50 μg/ml conforms with the observation of Kaufman and his associates (2), who, using embryo chick heart tissue embedded in a plasma clot, observed inhibi-

FIG. 3. A higher magnification of Fig. 2, illustrating cytoplasmic vacuoles. X 450.

tion of migration of fibroblasts with 10, 25, 50, and 100 μg/ml quantities of cortisone (free alcohol) and hydrocortisone and concluded that amount of inhibition was directly related to concentration of the steroids.

The formation of cytoplasmic vacuoles in fibroblasts grown together with cortisone or hydrocortisone has been observed by other workers. Paff and Stewart(7), who precipitated crystals of cortisone (free alcohol) by evaporation on coverslips before addition of nutrients and chick embryonic dermal fragments, reported that the first apparent reaction of the fibroblast to the hormone was formation of cytoplasmic vacuoles. Steen(1), using hanging drop cultures containing cortisone acetate, also observed cytoplasmic vacuoles in fibroblastic outgrowth from chick embryo heart. The presence of large fat vacuoles in an unusually early stage of growth was noted by Grossfeld and Ragan(3) in chick embryo cultures submitted to the action of soluble hydrocortisone.

The failure of cortisone or hydrocortisone to influence the mitotic index of rat fibroblasts or alter distribution of the 4 phases of mitosis accords with the work of Grossfeld and Ragan(3), who found no arrest of mitosis in any particular phase.

Summary. 1) The effect of cortisone acetate and hydrocortisone (free alcohol) on growth of L strain mouse fibroblasts was studied. 2) Addition of 10 μg/ml of either steroid to the culture fluid, after 11 days of incubation, resulted in a decrease in total cell count when compared with counts in control cultures. Hydrocortisone was the more inhibitory agent. 3) The effect of 3 concentrations of hydrocortisone (10, 20, and 50 μg/ml) on growth of fibroblasts over a 14-day period was established. The most marked inhibitory effect was produced during the first 7 days of culture. Fifty micrograms per ml were approximately twice as inhibitory as the lesser amounts of hydrocortisone. 4) Deviations from normal morphology of fibroblasts were seen in the steroid-treated cells. The cell processes were shortened, cells were swollen and flattened and tended to cohere in sheets. A great number of giant cells, often multinuclear or with nuclei irregularly shaped, were present. The cytoplasm contained many vacuoles.

The authors are indebted to Dr. C. Andrew L. Bassett for the photomicrographs.

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Phagocytosis of Periodate-Treated and Virus-Coated Red Blood Cells By White Blood Cells *in vitro*. (23224)

ZAHARA JERUSHALMY, ANDRÉ DE VRIES, ALEXANDER KOHN AND SHAUL KOCHWA
(Introduced by A. L. Olitzki)
Rogoff Clinical Research Institute, Beilinson Hospital, Petah Tiqva, and Israeli Institute for Biological Research, Ness Ziona, Israel.

Hemolytic anemia has been reported in virus diseases, such as infectious mononucleosis, virus pneumonia, Coxsackie A infection, influenza A infection and measles (reviewed by Dacie(1)). Several viruses are known to be adsorbed to red blood cells, and cause irreversible changes in their membrane(2). It has been shown that treatment of red cells with virus may render them antigenic and agglutinable by normal and immune serum(3, 4). Recently it was reported by Stewart, Petenyi and Rose(4) that canine erythrocytes treated with influenza virus had a shortened lifespan when infused into normal dogs. In some viral diseases, infectious mononucleosis, virus pneumonia and encephalitis due to poliomyelitis virus(5,6) leucocytes containing red cells have been found in the peripheral blood. Mabry and co-workers(7,8) demonstrated that treatment of human and rabbit erythrocytes with influenza or Newcastle disease virus increased their susceptibility to phagocytosis by rabbit spleen macrophages in tissue culture in the presence of serum opsonic factors.

The present study reports effect of influenza-virus treatment of human red cells on their subsequent susceptibility to phagocytosis by human white blood cells *in vitro*.

Methods. Leucocyte suspension. 5 ml samples of heparinized venous human blood were incubated at 37°C for 45 minutes in test tubes (100 × 13 mm) slanted at 45°, allowing the red cells to sediment. The supernatants were pooled and transferred into a siliconized (Dow Corning D.C. 200) test tube and centrifuged (1500 rpm for 30 seconds). The leucocyte sediment was washed twice with a 10-fold volume of phosphate-buffered saline (pH 7.2) and resuspended in the buffered saline to a concentration of about 1.5×10^7 leucocytes per ml. The leucocyte suspensions were kept at room temperature and used within 3 hours of drawing of the blood. *Red cell suspension.* The sedimented red cells were washed twice with buffered saline and a 2% suspension prepared. *Treatment of red cells with periodate.* To 1 ml of a 2% red cell suspension was added 1 ml of a 0.01 M sodium periodate solution in buffered saline. The mixture was kept at 4°C for 20 minutes and then 0.05 ml of 10% glucose was added to arrest the action of periodate. After 3 minutes at room temperature the cell suspension was centrifuged, the sediment washed with 2 ml buffered saline and resuspended in 1 ml of this medium. *Serum deficient in periodate-agglutinin.* Periodate-treated human red cells are agglutinated by compatible human serum(9,10). The periodate agglutinin responsible for this phenomenon was removed from the serum by repeated adsorption at 4° C for 15 minutes with periodate-treated red cells at a final concentration of 2% (generally 8 to 9 adsorptions were required). *Destruction of virus receptors on red cells by receptor destroying enzyme (R.D.E.).* Red cells were treated with R.D.E. according to Burnet and Stone(11). *Virus.* FMl strain of influenza virus in form of infected allantoic fluid (50th-80th chicken embryo passage) or red cell eluate in buffered saline was used. The virus was concentrated and purified according to the method of Francis and Salk (12). The hemagglutinin titers of the suspensions were determined in serial dilution of 1.5-fold steps with a 1% human O-red cell suspension. The virus suspensions were stored at 4°C as long as the titer did not change (not more than 5 days). *Adsorption, elution and inactivation of the virus.* For adsorption of the virus on the red cells, to be used in the phagocytosis experiments, a 2% suspension of red cells was incubated in the virus suspension for 20 minutes at 4°C. When desired, the virus suspension was eluted from the red cells as follows: the cells with the adsorbed virus were centrifuged in the cold, the supernatant was replaced with fresh phosphate buffered saline and the suspension incubated for one hour at 37°C. Subsequently the cells were thrown down, the supernatant was discarded and the cells resuspended for phagocytosis. The supernatants were titrated for presence of hemagglutinins, to insure that adsorption and elution were successful. Inactivation of the virus by heat was done by incubating the virus suspension for 30 minutes at 56°C. *Erythrophagocytosis experiment.* One drop of the red cell suspension was mixed with one drop of the leucocyte suspension and one drop of serum on a watch glass. The mixture was incubated in a moist chamber at 37°C. Smears were prepared from the sedimented cells and stained with Giemsa. The results were expressed as erythrophagocytic index, *i.e.* the percentage of granulocytes plus monocytes with at least one red cell engulfed. A total of 500 granulocytes plus monocytes was examined in fields where clumping of white cells and of red cells was minimal.

Results. Erythrophagocytosis of virus-treated red cells. Normal red cells or red cells onto which virus had been adsorbed, were not phagocytized by leucocytes in the presence of compatible (in regard to A, B agglutinin) serum, when incubated at 37°C for periods up to one hour. Similarly red cells from which the virus had been eluted or red cells treated with receptor destroying enzyme, were not phagocytized. In order to prevent elution of the virus from the red cells, during the phagocytosis experiment, three different methods were used. a) *Decreased temperature.* No phagocytosis of virus-coated red cells occurred on incubation of the erythrophagocytic system at 21°C for 20 minutes; at this temperature and time the elution of virus was negligible. At 21°C leucocytes phagocytized red cells in the presence of incompatible serum (erythrophagocytic index 12%). b) *Heat-inactivation of virus.* Red cells coated with heat-inactivated virus were not phagocytized. c) *Pretreatment of the red cells with periodate* (Table I). Red cells treated with periodate were readily ingested in the presence of compatible whole serum; in some experiments the erythrophagocytic index reached 40%. It was found that for the phagocytosis of periodate-treated red cells, the presence of serum was essential. The periodate-treated red cells in the erythrophagocytic system showed marked agglutination. Further it was found that serum made deficient in periodate agglutinin had lost its opsonic property for periodate-treated red cells. When serial dilutions of whole serum were made with either saline or serum deficient in periodate agglutinin, the agglutinating and opsonic activities disappeared at about the same dilution.

Adsorption of virus on periodate-treated red cells did not increase the erythrophagocytic index in the presence of whole serum, over that of red cells treated with periodate only; actually a decrease of the erythrophagocytic index was observed, probably due to massive clumping of the periodate-treated, virus-coated red cells, occurring in the presence of periodate agglutinin. However, in the absence of periodate agglutinin, coating with virus of the periodate-treated red cells induced marked erythrophagocytosis, while without virus periodate-treated red cells were ingested to a small extent only or not at all.

Disappearance of the virus in the erythrophagocytic system. When red cells (*not* treated with periodate) onto which virus was adsorbed, were incubated with leucocytes and serum for periods up to one hour at 37°C, no virus could be detected in the supernatant of such a mixture, although during this period elution of the virus from red cells proceeded. The disappearance of the eluted virus could be due to its adsorption on the leucocytes or to phagocytosis. The experiments of Boand, Kempf and Hanson(13) point to the second possibility.

Discussion. The observations show that neither the presence of influenza virus nor destruction of viral receptors on the red cell was sufficient to induce erythrophagocytosis by white blood cells *in vitro*. However when adsorbed onto periodate-treated red cells, the virus had a marked erythrophagocytosis promoting effect, manifest in the absence of periodate agglutinin. In view of the experiments of Nelson(14) on phagocytosis of bacteria adsorbed onto red cells, it seems quite feasible that in the erythrophagocytic system, containing reversibly adsorbed or heat-inactivated virus, the virus was phagocytized off the red cells. Nelson(15) had indeed suggested that virus adsorbed on red cells would be more readily phagocytized than virus free in suspension. It is possible that in the case of periodate-treated, virus-coated red cells, to which the virus is more firmly attached, the leucocytes try to engulf the virus, but cannot detach it from the red cells and ingest them both together. Final proof for such a mechanism may be possibly furnished by electron microscopy. Another explanation may be that the periodate-treatment and the adsorption of virus damage the red cells to an extent that the phagocytes ingest them as foreign bodies.

The role of specific antibodies in the phagocytosis of virus-coated red cells has not been satisfactorily elucidated. In all sera tested

TABLE I. Phagocytosis of Periodate-Treated, Virus-Coated Red Cells by White Blood Cells *In Vitro.*

Supplemental treatment of periodated red cells	Compatible serum	Erythrophagocytic index, % Exp. 1	Exp. 2	Exp. 3
None	Whole	26	39	38
Virus adsorbed at 4°C	"	19	30	20
None	Periodate-agglutinin deficient	0	3	4
Virus adsorbed at 4°C	*Idem*	11	20	21

by us a certain amount of influenza antibodies (hemagglutination-inhibition) was present. When such antibodies were removed by adsorption with virus-coated red cells the phagocytosis decreased. It was however subsequently found that the antibody adsorption resulted in removal of complement, too. The relative role of specific antibodies and of complement in promoting erythrophagocytosis is still under investigation.

The results obtained here with white blood cells differ from those obtained by Mabry and co-workers(7,8) with rabbit tissue macrophages in tissue culture, as macrophages readily ingested red cells to which influenza virus was reversibly attached. Whether this difference is due to the type of phagocyte or to the medium is not known. Nor is it known whether in the human body irreversible adsorption of virus to the red cell is required for the erythrophagocytosis observed in the peripheral blood in various diseases.

Summary. The presence of influenza virus or destruction of viral receptors on human red blood cells did not induce erythrophagocytosis by human white blood cells in presence of compatible serum *in vitro*. Influenza virus adsorbed onto periodate-treated red cells induced marked erythrophagocytosis in the presence of periodate-agglutinin deficient serum, while periodate-treated red cells without adsorbed virus were not phagocytized.

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Effect of Ornithosis on Experimental Fowl Malaria.* (23225)

Henry R. Jacobs
Department of Medicine, Northwestern University Medical School.

In a large series of immunological experiments with ducklings, heightened resistance to *P. lophurae* infections was found to be associated with mortality from an unknown cause. Ornithosis was isolated and identified as the cause of the unexpected mortality and as responsible for the heightened resistance to experimental malaria infections. The virus was isolated from lymph glands, spleens and from air sacs. The spleen was the most dependable source. The virus was identified as follows. It caused typical disease and histological changes in ducklings with great regularity. Liver and spleen were enlarged due to great expansion of the reticulo-endothelial system. Fibrinous exudates were present in the pericardial sac and in the air sacs. Typical organisms were always to be found in air sac membranes by the Machiavello stain. The virus would not pass a Seitz EK filter. Bacteriological studies in considerable detail disclosed no other organisms. Serological examination of the blood of recovered ducklings was positive for ornithosis-psittacosis, but this evidence is weakened by the fact that the disease was endemic in the laboratory flock. Finally, Dr. Karl Meyer was able to identify our organism as ornithosis. All this evidence, together with the unfailing infectiousness of standard preparations in young ducklings resulting in disease with typical gross and microscopic findings, makes it reasonable to accept the agent as that of ornithosis.

Methods. The virus was kept in passage by intraperitoneal transfer of suspensions of infected spleen. For immunological experiments a clear suspension was made from supernates of infected spleen suspensions by diluting with ice water and bubbling carbon dioxide through in the cold; the white precipitate was removed and suspended in saline to form a colorless, water clear preparation suitable for intravenous use and for filtration experiments. Ordinarily 20 ml of this refined suspension represented the material from one infected spleen; usually 2 ml was enough when given intravenously to young ducklings to cause typical moderately severe disease in 5-8 days.

Results. Ornithosis infections of graded severity were produced in young (under 6 days) ducklings by intravenous injections of the material described. Ten to 12 days later a challenge dose of 20 million living *P. lophurae* was given intravenously. Twelve experiments in which the original ornithosis infections were purposely severe are shown in resume in Table I. There is some mortality from ornithosis, but a very high resistance to malaria is also evident. Ten other experiments in which the original ornithosis infections were purposely milder are shown in Table II. In this group none of the ducklings died from ornithosis, but a high survival rate from malaria is again evident.

The results appearing in Tables I and II exemplify many others very similar in nature secured in this laboratory from experiments in which infectious material from blood, lymph glands, air sacs, fibrin and liver was given by routes other than the intravenous one (subcutaneous, intramuscular, oral, intraperitoneal). It is safe to say that if young ducklings are adequately infected with ornithosis by any route 7 or more days before challenge they will exhibit greatly heightened resistance to malaria, and that this increased resistance is associated with evidence of the presence of ornithosis. Enlargement of spleen

TABLE I. Young Ducklings Infected with Ornithosis Virus and 12 Days Later Challenged with Malaria. Moderately severe ornithosis. Combined results of 12 experiments.

	Test	Control
No. of ducklings used	156	66
" surviving ornithosis infection	140	
" " malaria challenge	135	6
% " malaria challenge	96.5	9.1
% having mild parasitemia	94	3

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TABLE II. Young Ducklings Infected with Ornithosis Virus and 10 Days Later Challenged with Malaria. Mild ornithosis. Combined results of 10 experiments.

	Test	Control
No. of ducklings used	130	52
" surviving ornithosis infection	130	
" " malaria challenge	126	4
% " malaria challenge	97	7.7
% having mild parasitemia	98	2

and liver (due to ornithosis) is always present in resistant ducklings.

Summary. 1) Ducklings infected with ornithosis 6 to 14 days before a challenge dose of *P. lophurae* malaria parasites exhibit a marked resistance to the challenge. 2) The resistance is associated with increase of the reticulo-endothelial elements in the enlarged liver and spleen due to the ornithosis infection.

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Oviduct Response to Estrogen and Progesterone in the Ring Dove (*Streptopelia risoria*).* (23226)

Daniel S. Lehrman and Philip Brody (Introduced by F. A. Beach)
Department of Psychology, Rutgers University, Newark, N. J.

Both antagonistic(1) and synergistic(2,3, 4) effects of progesterone and estrogens on weight of the oviducts of domestic hens have been reported. Mason(5) showed that the amounts and dosage ratios of estrogen and progesterone determined, in part, whether an antagonistic or synergistic effect would be observed. Furthermore, the 2 hormones appear necessary for normal oviduct development, as estrogen alone causes gland growth but progesterone in the presence of estrogen induces formation of secretion granules in the magnum(4).

In the course of a study of the hormonal induction of incubation behavior in the ring dove(6), behavioral effects of the hormones were found which suggested that the normal course of nest-building and incubating behavior depends upon the *successive* effects of estrogen and progesterone. The present study was undertaken to determine whether the effects of estrogen and progesterone on oviduct weights in this species are consonant with the hypothesis that progesterone is secreted after a period of estrogen secretion.

Methods. Sixty-three female ring doves (*Streptopelia risoria*), approximately 4 months of age, were selected for the experiment on the basis of direct examination of the ovary and oviduct through an incision in the abdominal wall. All had first and second phase ovules and undeveloped oviducts, and were thus not quite mature(4,7). The mean body weight was 148.7 g, S.D. 15.2 g. The substances used were injected (in 0.1 ml sesame oil) into the pectoral muscles on alternate sides from day to day, for 10 days. On the day following the last injection, the birds were killed by an overdose of pentothal sodium, and the oviducts removed and weighed. Groups of 9 birds each were treated as follows: 1) Control. 0.1 ml sesame oil daily x10. 2) Progesterone. 0.1 mg daily x10. 3) Estrogen. 0.4 mg diethylstilbestrol daily x10. 4) Progesterone + Estrogen. 0.1 mg progesterone plus 0.4 mg diethylstilbestrol daily x10. 5) Control primed. 0.1 ml sesame oil daily x8, followed by 0.1 mg progesterone plus 0.4 mg diethylstilbestrol daily x2. 6) Progesterone primed. 0.1 mg progesterone daily x8, followed by 0.1 mg progesterone plus 0.4 mg diethylstilbestrol daily x2. 7) Estrogen primed. 0.4 mg diethylstilbestrol daily x8, followed by 0.1 mg progesterone plus 0.4 mg diethylstilbestrol daily x2.†

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† Progesterone ("Lutocylin," Ciba) was supplied through the courtesy of Dr. Robert Gaunt of CIBA. Diethylstilbestrol was supplied through the courtesy of Dr. Aleck Borman of E. R. Squibb and Sons.

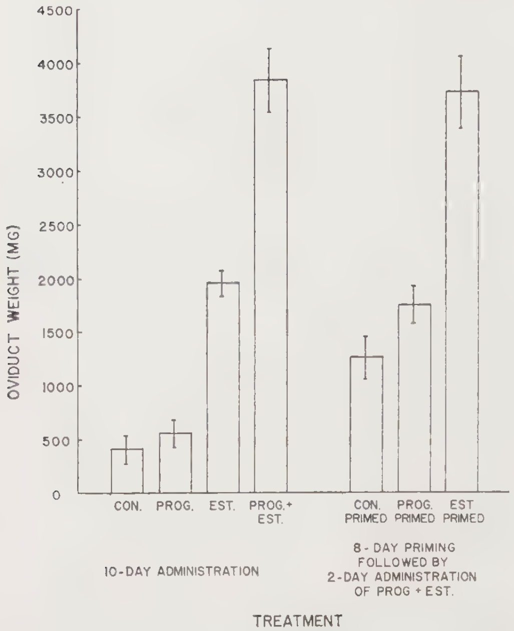

FIG. 1. Effect of hormone treatment on oviduct weights of ring doves. Length of vertical bars = 2 standard errors of mean (9 birds/group). Con. = Control (sesame oil). Prog. = Progesterone. Est. = Estrogen (diethylstilbestrol) (see text).

Results. The oviduct weights are presented in Fig. 1. Differences between all groups were tested for statistical significance by the Mann-Whitney U test(8), and the results of the comparison are shown in Table I.

Progesterone administration, in the dosages used, did not increase oviduct weights. Estrogen, however, significantly enhanced oviduct weight, and the combined administration of progesterone and estrogen caused a striking and significant increase over the effect of estrogen alone. These differences are easily discernible on gross examination of the oviducts. The dosage ratio of 4 estrogen/1 progesterone is one that had been reported by Mason(5) to have synergistic effects.

Priming for 8 days with estrogen, followed by 2-day administration of the estrogen-progesterone combination, caused as much oviduct growth as did 10-day administration of the combination. The weights of these oviducts were comparable to the maximum weights achieved in a normal breeding cycle. On the other hand, priming for the same period with progesterone did not significantly increase the effect of the 2-day administration of the 2-hormone combination, and caused much less oviduct growth than the 10-day administration of the combined dose.

Discussion. These data suggest that the normal growth of the oviduct, which reaches a sharp peak just after the laying of the first of the 2 eggs(9), could be accounted for by assuming the secretion of estrogen which causes moderate increase in weight, followed by secretion of progesterone for a short time, causing a sharp increase in weight.

There are other sources of evidence which support such a hypothesis of the ovarian picture in the nesting female ring dove. Fraps (10) reports that a single injection of progesterone into a laying hen will stimulate the premature release of an egg by the ovary. If progesterone is normally so involved in egg-laying in the ring dove, we might tentatively assume the occurrence of progesterone secretion just before ovulation of the first egg, which could account both for the ovulation and for the greatly increased oviduct growth which is observed at this time(9). Since the progesterone seems to stimulate ovulation via release of pituitary LH, however, there would still remain a number of questions concerning the initial stimulation of progesterone secretion.

Observations of nesting and incubating behavior suggest the same pattern. Estrogen injection has the same effect as does participation in courtship upon the behavior toward eggs subsequently introduced into the cage: it induces intensive nest-building, followed

TABLE I. P-Values for Comparison of Oviduct Weights of Groups of Ring Doves. Mann-Whitney U test. The first-named group in each pair had the larger mean oviduct wt.

Groups compared	P
Progesterone + estrogen *vs* estrogen primed	>.10
Progesterone *vs* control	>.10
Estrogen " "	<.002
Progesterone + estrogen *vs* control	"
Estrogen *vs* progesterone	"
Progesterone + estrogen *vs* progesterone	"
" + " " estrogen	"
Progesterone primed *vs* control primed	>.10
Estrogen " " " "	<.002
" " " progesterone primed	"

after 1-3 days by incubation(6,12). Progesterone injection has the same effect as does participation in courtship and nest-building: it induces immediate incubation(6,11). This suggests that the normal cycle may include a period of nest-building (associated with estrogen secretion), which induces a subsequent period of incubation (the beginning of which is associated with progesterone secretion).

Brant and Nalbandov(4) state that, since androgen also has synergistic effects with estrogen on oviduct growth, and since androgen has been found in the fowl ovary(13), the physiological substance involved in oviduct growth may be androgen rather than progesterone. While this is possible, several considerations suggest that the physiological involvement of progesterone should be further explored: 1) Progesterone has been found in the blood of hens and roosters(14,15); 2) Androgen stimulates male sexual behavior, which is antagonistic to the broodiness induced by progesterone and normally occurring at the time of egg-laying and of maximum oviduct growth(16,17); 3) Although testosterone and progesterone both augment the effect of estrogen on oviduct weight in hens, the effect on female sexual behavior in the same birds is that progesterone augments the effect of estrogen, while androgen does not(2); 4) Progesterone causes immediate incubation in all doves treated, while androgen does not(9). Progesterone, unlike androgen, can thus account for both the behavioral and the morphological changes which take place at about the time of egg-laying.

Summary. 1. Adolescent female ring doves (*Streptopelia risoria*) were injected with progesterone, estrogen or a combination of the two. Progesterone did not increase oviduct weights over the controls. Estrogen caused moderate increase, while the maximum increase was caused by synergism between estrogen and progesterone. 2. Eight-day priming with estrogen, followed by 2-day treatment with the estrogen-progesterone combination, caused oviduct growth as great as that caused by the 10-day treatment with the combined dose. On the other hand, priming with progesterone did not significantly augment the effect of 2-day treatment with the combination. 3. On the basis of these data and of a discussion of the mechanisms of nest-building and incubation behavior, it is suggested that the normal cycle includes a period of estrogen secretion (concurrent with nest-building) followed by progesterone secretion (associated with egg-laying maximal oviduct growth, and incubation behavior).

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Biosynthesis of Liver and Tumor RNA Ribose in the Tumor-Bearing Rat. (23227)

PHILIP FEIGELSON AND PAUL A. MARKS
Departments of Biochemistry and Medicine, College of Physicians and Surgeons, Columbia University, New York City.

Rapid glucolysis with the aerobic accumulation of lactic acid is a prominent metabolic aberration manifested by tumors(1). It is therefore of interest to ascertain whether other alterations in tumor carbohydrate metabolism may exist. Towards this end a study has been undertaken on the biosynthesis of the pentose, ribose. The recent development of isotopic tracer and degradative technics(2) has permitted *in vivo* studies of the various alternate pathways of pentose biosynthesis in the normal rat(3,4). These studies have now been extended to the tumor-bearing rat in an attempt to gain insight into the biosynthetic pathways of RNA in the tumor, as well as to determine the effect of the tumor on nucleic acid pentose synthesis in the liver of the host animal.

Methods. Adult male Wistar rats weighing between 75 and 100 g were inoculated intraperitoneally with approximately one million Yoshida ascites tumor cells. Five days after inoculation food was withdrawn from all animals and on the sixth day the animals were administered by stomach tube 1 ml of 25% glucose/100 g body weight. One hour later each animal received a subcutaneous injection of 20 μc of glucose-2-C^{14} (supplied by Dr. H. I. Isbell of the National Bureau of Standards). After a 6 hour isotope incorporation period the animals were sacrificed by a blow on the head and then exsanguinated. The ascitic tumor cells were removed with a hypodermic syringe and the peritoneal cavity washed with saline to collect remaining tumor cells. The livers were then removed and further washed with saline to remove adhering tumor cells. Employing technics previously described(4), the tumor and liver RNA were separately isolated, the RNA purine-bound ribose was liberated by acid hydrolysis, freed of ionic contaminants with ion exchange technics and then further purified employing paper chromatographic procedures. The isolated liver and tumor RNA ribose was degraded using *Lactobacillus pentosus*(5,6) followed by chemical degradation technics; each ribose carbon atom was thus individually isolated as $BaCO_3$(7). The specific activities of the $BaCO_3$ samples thus obtained were estimated with either a windowless flow counter or a liquid scintillation counter.

Results. The results of duplicate experiments are depicted in Table I as the per cent specific activity in a given carbon atom relative to ribose carbon atom 2. The liver RNA ribose of the tumor-bearing rat presents an isotope distribution pattern that is conspicuously different from that of the control rat bearing no tumor. Whereas the bulk of radioactivity in the liver ribose of control animals is in carbon atom 2, with considerable activity in carbon one and little or no activity in ribose carbon atoms 3, 4, and 5, the radioactivity in the liver ribose of the tumor-bearing rats is widely dispersed throughout the ribose molecule, with considerable radioactivity present in carbon atoms 3, 4, and 5. In the tumor, however, ribose carbon atom 2 exhibits the highest specific activity; the remaining activity is distributed essentially equally throughout the rest of the ribose molecule.

Discussion. The conspicuous finding in this study is the extensive randomization of the C^{14} throughout the carbon chain of ribose in both the tumor and the liver RNA of the tumor-bearing animal. This effect is more marked in liver RNA ribose than in the tumor RNA ribose. The distribution of the C^{14} labeling pattern in liver RNA ribose of the Yoshida tumor-bearing rat is thus in sharp contrast to the labeling pattern previously observed in the normal rat liver RNA ribose (4). This alteration in labeling pattern may be subject to 2 alternative interpretations: (1) The tumor exerts an influence on liver metabolism; or (2) rapid metabolism by the

TABLE I. Incorporation of Glucose-2-C^{14} into Liver and Tumor RNA Ribose of Yoshida Ascites Bearing Rats.

	Ribose carbon No.				
	1	2	3	4	5
Tumor	36.2*	100	40.6	45.4	30.8
	35.2	”	45.4		
Liver	100	”	94.4	93.5	84.4
	90.5	”	72.7	93.6	72.7
Control liver	34.4	”	5.3	0	0
(Tumor-free rat(4))	23.6	”	2.0	0	0

* Values expressed as % of specific activity in C_2.

tumor of the administered glucose-2-C^{14} results in a randomization of label throughout the hexose; the observed pattern of ribose labeling in the liver resulting from normal pentose synthesis by the liver, utilizing, however, a hexose precursor in which all carbon atoms are labeled. This latter alternative is supported by the recent finding that glucose-2-C^{14} administration to tumor-bearing mice resulted in liver glycogen containing C^{14} distributed throughout all carbons of the hexose (8). These findings have been interpreted as indicating that the tumor caused an extremely active reversible conversion of the administered C^{14} glucose through the Embden-Meyerhoff and tricarboxylic acid cycles resulting in migration of the C^{14} from its original position to other locations in the glucose molecule. Whether these alterations are due to the tumor *per se* or to the indirect influence of the presence of the tumor on the metabolism of other tissues remains obscure.

While uncertainty as to the labeling pattern of the hexose precursor of ribose in tissues of tumor-bearing rats precludes precise interpretations of the pathways of pentose biosynthesis, the preponderance of label in carbon 2 of ribose would seem to eliminate decarboxylation of hexose as the dominant reaction in pentose biosynthesis(4).

Summary. Pathways of pentose biosynthesis in the liver and tumor of control and tumor bearing rats have been studied employing glucose-2-C^{14} as the isotopic precursor. Presence of the tumor in the animal caused marked alteration in the labeling pattern, of liver RNA ribose, with the C^{14} being distributed almost equally throughout the carbon chains. However, tumor RNA ribose is characterized by greater isotopic asymmetry with carbon atom 2 being most radioactive. Possible metabolic mechanisms underlying these observations are discussed.

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Plasma Catechol Amines in Psychiatric Patients. (23228)

Joseph Reilly and Peter F. Regan III

Department of Psychiatry, Cornell University Medical College and New York Hospital (Payne Whitney Psychiatric Clinic).

Interest in the determination of plasma concentrations of catechol amines has increased since the development of sensitive fluorometric methods by Lund(1) and Weil-Malherbe and Bone(2). The ethylenediamine method of Weil-Malherbe and Bone is more sensitive but less specific to epinephrine and norepinephrine than the Lund method. It has been used to follow the changes in blood levels of catechol amines in psychiatric

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TABLE I. Concentration of Catechol Amines in Plasma of Psychiatric Patients.

	Subjects	Conc. of amine (μg/liter)* Epinephrine	Norepinephrine
Patients	48	1.2 ± .10	3.3 ± .24
Controls	27	1.2 ± .09	3.0 ± .25
P value		>.8	>.4

* Mean ± S.E.

clinics following therapeutic treatment(3,4). The ethylenediamine method has also been found reliable in measuring the altered levels of circulating norepinephrine and epinephrine found in pheochromocytoma(5) and a modification of the method(6) has been used to follow changes in amine levels in patients undergoing various surgical procedures(7). Several workers have used the ethylenediamine method to measure the changes in circulating catechol amines after anesthetic(8,9) and other drug actions(10,11,12) in experimental animals.

It is the purpose of this paper to report the levels of circulating catechol amines, with fluorescent spectral characteristics similar to epinephrine and norepinephrine, in psychiatric patients of various diagnostic categories.

Methods and chemical procedure. The catechol amine content in plasma was measured by the method of Weil-Malherbe and Bone(1). The test was started immediately after the blood was drawn. All of the chemical tests were performed by the same person. Slight modifications were made in the methodology. The amines were adsorbed from heparinized plasma by shaking with alumina washed free of fluorescent material. The alumina used was the non alkaline type (Woelm brand) recommended by Aronow. The use of double distilled water and ethylenediamine redistilled daily resulted in a minimum of fluorescence in the blanks. The fluorescence of standard solutions of crystalline epinephrine and norepinephrine were always compared to the unknown samples. Recoveries averaged 90% for both amines.

Fluorescence of ethylenediamine-coupled amines was measured in a Farrand fluorometer Model A, equipped with a photomultiplier tube. The primary filter system was composed of Corning filters numbers 5113 and 3389, which passed the 436 mμ line. One secondary filter consisted of Corning numbers 5433 and 3384, which passed a band in the region of 510 mμ and the other secondary filter was a Corning number 2418 with a cut off at approximately 600 mμ. *Psychiatric Evaluation.* Patients were selected from the inpatient service of the Payne Whitney Psychiatric Clinic and represented a range of diagnostic categories and of emotional disturbance. Evaluation of the patient's diagnostic and emotional status at the time of testing was made by one of the authors (P.R.) who was familiar with all of the patients. Control subjects were hospital employees (physicians, nurses, laboratory and clinical workers) who showed no clinical evidence of psychiatric illness. In all, 48 patients and 27 controls were studied. Although most patients have received many repeated studies, only the first observations are reported here.

Results. Twenty-one of the patients studied had schizophrenic reactions, 19 affective disorders, 4 paranoid reactions, and 4 psychoneurotic reactions. In 48 samples of plasma, obtained from patients and tested for catechol amines of the epinephrine and norepinephrine type, considerable variation in the plasma amine level was observed. The range of epinephrine values in 48 patients varied from zero to 2.5 μg/liter of plasma, while the range of norepinephrine values varied from 0.1 to 8.6 μg/liter of plasma. The range of epinephrine values in 27 controls varied from zero to 2.5 μg/liter of plasma and the range of values for norepinephrine varied from 0.4 to 5.6 μg/liter. These data, tabulated in Table I, showing a comparison between catechol amine levels of patients and controls, were obtained within the same time period. No significant difference was found between the

TABLE II. Concentration of Catechol Amines in Plasma of Patients of Different Diagnostic Categories.

Diagnostic category	No. of patients	Conc. of amine (μg/l)* Epinephrine	Norepinephrine
Schizophrenic	21	1.1 ± .17	3.3 ± .39
Affective reaction	19	1.3 ± .16	3.3 ± .42
Paranoid "	4	.75 ± .22	3.3 ± .47
Psychoneurotic	4	1.5 ± .40	2.3 ± .14

* Mean ± S.E.

TABLE III. Fluorescence of Possible Interfering Substances in Weil-Malherbe and Bone Method.

Compound	Conc. μg/100 ml	% of fluorescence produced by 1 μg/100 ml of Epinephrine	Norepinephrine
Dihydroxyphenylalanine	100	15	0
3 hydroxytyramine	1	60	100
Tyramine	5000	20	30
5 hydroxytryptamine	200	0	40

data of the patients and controls.

A further analysis was made of the patient group in which the patients were considered as to diagnostic category (Table II). A test of significance was made between each diagnostic category as compared to the controls. The only significant difference observed was that of the norepinephrine levels in the psychoneurotic group as compared to the control norepinephrine levels. A "P" value of .02 was found but the sample of 4 psychoneurotics is to be extended before we positively assign significance to this category.

Interfering Compounds. Three of the compounds considered to interfere with the ethylenediamine test were compared to 1 μg % solutions of epinephrine and norepinephrine (Table III). These compounds, dihydroxyphenylalanine, tyramine and 5 hydroxytryptamine showed interference qualitatively similar to that reported by Weil-Malherbe and Bone(2). The fourth compound, 3 hydroxytyramine, was found to interfere with both epinephrine and norepinephrine in equivalent concentrations. Our data are in agreement with those of Valk and Price(13) that this compound, if present in plasma, could seriously interfere with the ethylenediamine method.

Discussion. The ethylenediamine method used to determine the level of circulating catechol amine has been criticized by Valk and Price(13). They indicate that the ethylenediamine method is not specific to the amines, particularly norepinephrine, and possibly measures a degradation product of 3 hydroxytyramine metabolism such as dihydroxyphenylacetic acid. Nevertheless, the method does reflect changes in catechol amine content of the epinephrine and norepinephrine type in cases of pheochromocytoma(5). The ratio of these amines in the patients, as measured by the Weil-Malherbe and Bone method, is in agreement with the observations of Holton (14), Engel and Euler(15) and Goldenberg and Rapport(16). Furthermore, it can be demonstrated with this method that the catechol amine level slowly returns to normal values following surgical ablation of the tumor (17). We have also observed that a patient with pheochromocytoma having preoperative levels of 3.0 and 17.5 μg/liter of epinephrine and norepinephrine, respectively, gave values of 0.8 and 3.2 μg/liter 2 weeks post operatively. Thus it appears that the ethylenediamine method gives measurements which follow the clinical picture in cases of pheochromocytoma.

Experiments devised to determine a constant quality or quantity in the interfering substances have not been successful. The interference can be due to 3-hydroxytyramine, as indicated by our test (Table III) and that reported by Valk and Price(13). It remains to be demonstrated, however, that this compound or any other dihydroxy catechol derivative will persist in plasma. At this time, therefore, it would seem best to accept the conservative evaluation of the Weil-Malherbe and Bone method which was proposed by Hammond *et al.*(7). They state that the values reported by them as epinephrine and norepinephrine may include other compounds and that these values may be legitimately accepted as maximal concentrations of plasma epinephrine and norepinephrine. It is probable that the absolute values for these amines are lower than reported by the ethylenediamine method; nonetheless, the method has been useful in following fluctuations in catechol amine content.

The data presented in this report demonstrated that a group of psychiatric patients did not maintain blood levels of catechol amines that differ in general range from the blood levels of "normal" individuals. The findings are in agreement with those found in a study of surgical cases(7), and are in keeping with studies of most physiological variables in psychiatric patients. Most studies, however, have indicated that significant findings depend on the use of repeated examinations of the individual, in different situations (18-21). Our incomplete series of multiple testings indicate that this finding applies to blood catechol amines, and warrant further exploration with this method.

Summary. The concentrations of catechol amines with fluorescent absorption spectral characteristics similar to epinephrine and norepinephrine were determined in patients and hospital personnel in a psychiatric hospital. No significant differences were found in blood levels of catechol amines in these two groups. There were also no significant differences observed in the catechol amine levels among the various diagnostic categories within the patient group. L dihydroxyphenylalanine, tyramine, and 5 hydroxytryptamine do not appreciably interfere with the ethylenediamine method. 3 hydroxytyramine had a fluorescent potential equivalent to that of epinephrine and norepinephrine when these compounds were compared in the ethylenediamine method.

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Electrophoretic Patterns in Acute Acquired Hemolytic Anemia.* (23229)

LEON N. SUSSMAN AND JAMES B. SANG
Laboratory of Beth Israel Hospital, N. Y. City.

In the study of the immunohematology of acute acquired hemolytic anemia, the nature and behavior of the serum proteins attracted attention. An abnormal distribution of the protein fractions was suggested by the greatly accelerated sedimentation rate, autoagglutination, and high antibody titer. The only reference to this subject in man is a short note by Hansen(1) in which 3 of 4 cases of acquired hemolytic anemia are reported to have had elevated gamma globulins. Boyd has summarized the results in excess antibody production in animals(2).

Material and methods. Eight cases of ac-

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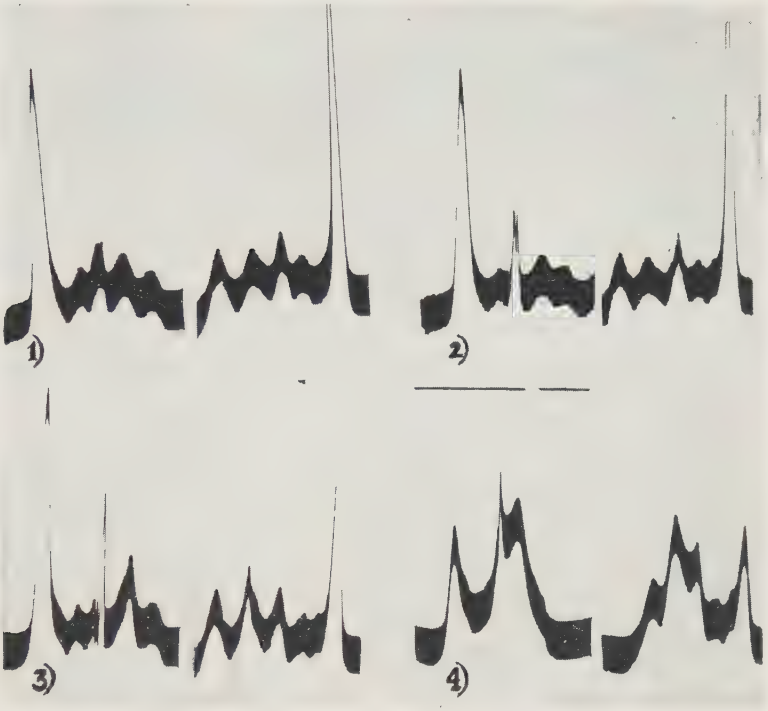

FIG. 1. Electrophoretic patterns in illustrative cases.

quired hemolytic anemia in adults with positive anti-human globulin tests were studied. This group consisted of 4 cases of primary hemolytic anemia, 2 cases of acquired hemolytic anemia secondary to lymphomas (one of lymphatic leukemia and one of lymphosarcoma) and 2 cases secondary to metastatic carcinoma with liver involvement and jaundice (Table I). The serums of these patients were examined electrophoretically by the Tiselius(3) technic in the following manner: 1 ml of clear serum was diluted with 2 ml of veronal buffer (pH 8.6) and ionic strength 0.1 u. The diluted serum was dialyzed for 30 hours against 450 ml of the buffer at 4°C. Analyses were made in the Perkins-Elmer electrophoresis apparatus model 38. The length of the run was 4200 seconds and the patterns were enlarged and calculated from the areas under curves with planimetry. The chemical analysis was performed by using 1 ml of clear serum for determination of total protein by the method of Looney and Walsh (4).

Results. Table I presents the findings obtained by both electrophoretic and chemical analyses. Fig. 1 demonstrates the pattern obtained in 1) normal; 2) patient AS (case #4) demonstrating an extreme beta globulin peak; 3) patient DS (case #6a) demonstrating an extreme gamma globulin peak; 4) patient AL (case #7) demonstrating an extreme beta and gamma globulin peak with low albumin.

Discussion. The major variations from normal appear in the increased combined beta globulin and gamma globulin fractions, as well as in low total albumin (expressed as g%). As shown in the table the increase in either fraction did not relate to the etiology, specificity of the antibody, or the titer. It has been shown that the components which migrate as a unit with the beta or gamma globulin consist of several distinct substances, separable by sedimentation constants and immunological technics(5). The exact component that varied in each case has not been determined. In fact, Vaughn(6) has recently presented evidence that some auto-antibodies were gamma globulins, but others were of

TABLE I. Composition of Serum Proteins Expressed in % of Total Protein (upper figures) and in g/100 ml (lower figures).

		Diagnosis	g %	Antibody & titer	Albumin	Alpha 1	Alpha 2	Beta	Gamma
		Normal		None	55-65%	4-7%	8-10%	11-14%	11-16%
					4-5 g%	.3-.5	.5-.7	.8-1.0	.8-1.2
1.	Y.F.	Idio.		Panagglutinin	60.8	5.	5.8	14.6	13.8
		Total	5.48		3.31	.27	.32	.80	.75
2.	S.D.	Idio.		Cold 4000	53.3	6.3	8.5	12.8	19.1
		Total	6.24		3.33	.39	.53	.80	1.19
3.	S.K.	Idio.		Anti rh' & K	43.4	8.1	11.3	17.2	20.
		Total	7.27		3.16	.59	.82	1.25	1.45
4.	A.S.	Idio.		Pan	56.3	6.0	6.2	17.4	14.1
		Total	6.95		3.91	.42	.43	1.22	9.7
5.	H.K.	Lymph. leuk.		Pan	58.4	8.8	11.1	17.8	3.9
		Total	6.28		3.67	.55	.70	1.12	.24
6. a)	D.S.	Lymph. Sa.		Cold 200	53.6	5.5	6.1	12.2	22.6
		Total	7.12		3.82	.39	.43	.87	1.61
b)	D.S.	Post-splenectomy		Cold 800	49.2	8.2	14.6	13.5	14.5
		Total	6.75		3.32	.55	.99	.91	.98
7.	A.L.	Meta-Ca.		Cold	20.9	5.2	7.8	24.1	42.0
		Total	6.20		1.30	.32	.48	1.49	2.61
8.	J.C.	Meta-Ca.		Cold 32	22.2	7.5	13.7	24.7	31.9
		Total	3.86		.86	.29	.53	.95	1.23

non-gamma globulin material and that some actually appeared to be of 2 components.

The very profound variations from normal found in Cases 7 and 8 were probably caused by the disturbance in the major protein fraction due to the severe liver involvement. Both of these patients, as can be seen, had reversal of the albumin-globulin fractions which completely masked the relatively minor influence of the auto-antibody present.

The mobility of iso- and hetero-agglutinin between beta and gamma globulin has been reported by others(7). The findings presented here would seem to verify that a true quantitative increase in these fractions does occur in the presence of excess unusual antibodies.

Summary. Electrophoretic and chemical study indicate that auto-antibodies, as found in acquired hemolytic anemia, produce a measurable quantitative increase in beta and gamma globulin fractions.

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Physical Activity, Serum Cholesterol and Other Lipids in Man.* (23230)

HENRY LONGSTREET TAYLOR, JOSEPH T. ANDERSON AND ANCEL KEYS
Laboratory of Physiological Hygiene, School of Public Health, University of Minnesota, Minneapolis.

Anderson, Lawler and Keys(1) have proposed the hypothesis that, other things being equal, the serum cholesterol concentration in man is determined by the fat transport load imposed on the blood per unit of circulation. The transport of fat from intestines to liver and from liver to fat depots is thought to be primarily involved. The transport of fat from the fat depots to the point of metabolism appears to be less directly concerned because the serum cholesterol concentration falls during negative calorie balance in spite of the fact that a great deal of fat is mobilized from the fat depots in this situation(2,3). The hypothesis explains: 1) The increase in serum cholesterol concentration observed on high fat diets and in overnutrition with a positive calorie balance; 2) The stability of serum cholesterol during calorie equilibrium and a constant fat intake and 3) The decrease in serum cholesterol on low fat diets and in negative calorie balance. The role of physical activity in this scheme needs clarification. The acute effects of physical activity have been studied in connection with dietary cholesterol loading(4) and it has been shown that when calories and activity are increased to the same extent so that calorie equilibrium is maintained, no increase in serum cholesterol concentration results(5). However, in the latter work only 3 subjects were studied, the quantity of fat in the diet was held constant while the physical activity was increased so that the proportion of calories represented by fat metabolism decreased sharply, and calorie equilibrium was not exact. It is of interest to discover whether increasing the fat content of the diet in proportion to the calories consumed by physical activity will affect the serum cholesterol concentration. This paper presents data bearing on this point.

Subjects, conditions and methods. The subjects were 9 clinically healthy university students who volunteered to participate in the experiment. The mean age was 23.3 years with a range of 21 to 28 and the mean relative weight was 108.3 with a range of 93 to 128. None of the men was excessively fat or thin. Two of the 9 played in intramural basketball but the others did not take part in organized sports.

All meals were prepared and measured in the laboratory and 2 meals a day were served in the laboratory dining room. Box lunches consisting of sandwiches, fruit and milk were carried by the subjects and eaten outside of the laboratory. All food items were served as weighed portions and the rejections were tabulated and used in calculating the composition of the diet. The diet was designed to approximate the current American practice of using liberal quantities of meat, milk, butter and eggs. It was fully adequate in vitamins and minerals. The calorie, fat and protein content are presented in Table I.

During the period of increased physical activity, the men walked for 2 hours a day on the motor driven treadmill at 3.5 miles per hour on a 10% grade in an air conditioned room in which the temperature was held constant at 78°F with a relative humidity of 50 to 60% saturation. Body weights were measured each morning before breakfast without shoes and coat. *Oxygen consumption* during work was determined by collecting expired air in a spirometer and analyzing an aliquot sample for CO_2 and O_2 concentrations in a Haldane gas analysis apparatus. *Blood* was drawn in the morning before breakfast. The total serum cholesterol was determined by a modification(6) of the method of Abell *et al.* (7). The cholesterol associated with the α and β lipoprotein was determined by cold ethanol fractionation with a modification of the procedure of Russ, Eder and Barr(8). For the determination of *lipid phosphorus*, serum samples were extracted with Bloor's

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TABLE I. Composition of Diets employed in the 2 sedentary periods and the physically active period.

Diet		Cal./day	Protein g/day	Protein % of cal./day	Fat g/day	Fat % of cal./day
Sedentary	Mean	3257	105	13.0	155	42.7
	St. dev.	78	4.6		3.6	
Physically active	Mean	4189	135	12.9	201	43.2
	St. dev.	79	8.3		5.1	

mixture and the dried extract was further purified by a second extraction with chloroform. The phosphorus was then determined following the procedure outlined by Brandt (9). Dole's method(10) was used to determine the concentration of non-esterified fatty acids.

Results. The general plan of the experiment was to have the men eat the standard sedentary diet for 2 weeks (S1 and S2, designated period I). This was followed by a transition period of 2 weeks in which the men increased their time spent walking on the motor driven treadmill from one-half to 2 hours a day (A3 and A4). Treadmill walking of 2 hours daily was then maintained for 2 weeks (A5 to A6, designated period II) and this was followed in turn by a week on the original diet (S7, designated period III) with no treadmill walking.

The body weights for the last 3 days of each week were averaged for each man. There was no significant variation in the means. At the beginning of the experiment the mean weight was 171.0; at the end of the exercise period, 171.0; and at the end of the second sedentary period, 170.8.

The results of the analyses of the blood lipids are presented in Table II. There were no significant differences in the group means of total serum cholesterol or beta lipoprotein cholesterol in the 3 periods studied. The variability between periods I and II was small while that between periods I or II and III is noticeably larger. The lipid phosphorus sustained a significant decline at the end of the activity period which was recovered after a week of sedentary existence. The nonesterified fatty acids tended to decline continuously during the entire 3 periods and the conventional T test for paired variates showed that none of the differences in the means approached statistical significance. The diet during the activity period provided an increase of 932 calories. The mean oxygen consumption of the group while walking on the treadmill was found to be 2.240 L/min. with an R.q. of 0.83. Two hours of work at this rate accounts for 1280 calories. In planning the experiment an allowance of 300 calories was made for basal metabolism and activities that would have been pursued dur-

TABLE II. Concentration of Cholesterol, Beta Lipoprotein Cholesterol, Phospholipid and Non-Esterified Fatty Acids (NEFA) of Subjects in the Several Periods. Figures in the table are means of duplicate determinations made in serum obtained from subjects on the last 2 days of designated period. All figures are in mg/100 ml of serum except NEFA which is expressed as micro equivalents of fatty acid/l of serum and Beta lipoprotein cholesterol which is in % of total.

	Serum cholesterol			Beta lipoprotein chol.			Phospholipid			NEFA		
	I	II	III	I	II	III	I	II	III	I	II	III
D	160	174	181	79.4	72.4	75.1	146	132	157	419	478	242
H	203	197	212	78.8	82.2	77.3	176	143	184	406	392	473
M	192	188	193	57.2	77.7	76.7	169	144	167	783	442	471
O'	144	152	179	77.1	83.6	82.7	123	129	149	423	340	256
Ol	116	122	132	80.2	72.1	77.3	113	106	128	560	442	596
P	163	159	125	79.8	82.4	76.8	136	145	110	720	1006	460
Q	147	146	140	74.2	75.3	82.1	142	130	140	439	372	525
R	150	147	144	83.3	87.8	90.1	134	118	137	475	342	394
S	150	154	148	77.3	74.7	77.0	131	121	127	345	344	320
Mean	158.3	159.8	161.5	76.3	78.7	79.5	141.1	129.7	144.3	507.7	462.0	415.2

ing the 2 hours which were occupied by treadmill work. The weight stability of the subjects during 7 weeks of the experiment suggests that this estimate was not far from correct.

Discussion. Since the oxygen intake required by the work task employed here is approximately 60% of the maximal oxygen intake found in young men of similar age and background(11), the work was entirely aerobic. The data presented by Asmussen and Nielsen(12) suggest that the cardiac output in treadmill walking of this intensity lies between 16 and 20 liters per minute or 3 to 5 times the basal rate. Circulation to the muscles is certainly increased by a larger factor since circulation thru the muscle at rest is probably only 20% of the resting cardiac output. Circulation to the muscles in the work conditions employed here is adequate to compensate for any increased fat transport to muscles that the lipoprotein cholesterol complex might take part in.

The work presented here is in agreement with the initial hypothesis that serum cholesterol concentrations are determined by the fat transport load from the intestine to the liver and to the fat depots which in turn is influenced by the circulation rate. In spite of the fact that the amount of fat eaten by the subjects increased by 46 g a day no increase in the level of serum cholesterol was observed, presumably because the circulation was increased in proportion to the additional fat load.

Mann *et al.*(5) studied 3 men who increased their calorie intake by roughly 3000 calories or 50%. The fat content of the diet was held constant so the proportion of calories provided by fat fell from 50 to 51% of the total to 24.7 to 25.2%, a reduction which might be expected to reduce serum cholesterol levels by as much as 20 mg per 100 ml of serum if activity and calories were constant (13). Heroic efforts were made by the subjects in this experiment to increase their energy expenditure to the level of roughly 6000 cal. a day. In spite of this increase in activity all men gained from 3 to 5 lb of weight suggesting that the positive calorie balance might be as high as 12,000 to 14,000 calories. The serum cholesterol levels either showed a downward trend or no change. The interpretation of these results is confused by the increase in body weight which, by itself, would tend to increase serum cholesterol levels(1). In the first 5 weeks of overnutrition, the subjects studied by Anderson *et al.*(1) gained 1.6 kilos of weight while their serum cholesterol concentrations increased by 20.3 mg per 100 ml of serum. The results reported by Mann *et al.*(5) might well be summation of two opposing factors.

Summary and conclusions. The effect of increasing the level of daily physical activity on the serum cholesterol concentrtaion was studied in 9 clinically healthy University students. The physical activity was increased by walking on the motor-driven treadmill at a rate which required 1280 cal. for 2 hours. The caloric intake was increased by 900 cal. but the proportion of calories derived from fat was held constant. There was no change in the serum cholesterol concentration. It is concluded that the results are consistent with the hypothesis that the serum cholesterol concentration is related to the proportion of total calories derived from fat.

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Strontium Metabolism and Strontium-Calcium Discrimination in Man. (23231)

C. L. COMAR, R. H. WASSERMAN, SVEN ULLBERG,* AND G. A. ANDREWS
Medical Division, Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn., under contract with U. S. Atomic Energy Com.

The need for a better understanding of the behavior of strontium in man has arisen because of development of nuclear weapons and potential applications of atomic energy. Radioactive strontium, disseminated worldwide, eventually finds its way into accessible calcium reservoirs of the biosphere (of which the human skeleton is one) and tends to attain steady state movement between such reservoirs. Information is needed to permit prediction of levels to be attained in the human population in terms of the amount falling out upon the earth or becoming incorporated in the diet. Since on a long-term basis the human burden will result from strontium contamination of dietary calcium, considerable advantage is gained by studying the comparative behavior of these two elements. Knowledge of the manner in which various physiological processes affect retention of strontium and calcium in the body may lead to ways in which the comparative retention can be modified at will. It is of practical importance to determine the extent to which the urine or blood levels can be used to estimate the body burden or over-all intake of radioactive strontium.

The world-wide distribution of strontium 90 has been reviewed by Libby(1) and Kulp *et al.*(2). The metabolism of strontium in laboratory and domestic animals has been extensively studied(3-6), and the behavior of intravenously injected strontium 85 in human subjects has been reported by Spencer *et al.*(7). Harrison *et al.*(8) have followed, in normal man, the course of stable strontium given in amounts (20 to 250 mg) that allowed analysis by radioactivation. These findings have been reviewed and compared with animal data by Comar and Wasserman(3).

The long-term problem is concerned with the behavior of essentially carrier-free radiostrontium ingested over an extended period with each meal. Therefore, in the present work, emphasis has been given to experiments that simulate this situation, rather than to the usual single parenteral dose-type experiment often employed in metabolic studies. Data are presented on the behavior of strontium 85 in man after single and daily feeding, and on the strontium-calcium discrimination as shown by daily feeding of strontium 85 and calcium 45 in double tracer studies.

Methods. These studies were done with chronically ill patients having leukemia or widespread malignant neoplasms. Carrier-free strontium 85 (cyclotron produced from rubidium; 65 day half life; no beta rays; 0.013 Mev X ray, 0.513 γ ray) and high specific activity calcium 45 were used. The levels of Sr 85 employed ranged from 5 to 20 microcuries for single dosage studies and from 1 to 3 microcuries per meal for the daily feeding experiments. In the double tracer studies, Ca 45 was given at about twice the microcurie amounts of Sr 85. The usual procedures for handling of samples and radioassay were employed(9) except as noted. The Sr 85 was counted with a well-scintillation crystal. Double counting was accomplished by scintillation measurement of the sample itself or of an aliquot of ash solution for Sr 85, and

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* W. K. Kellogg Foundation Fellow from Kungl. Veterinarhogskolan, Stockholm.

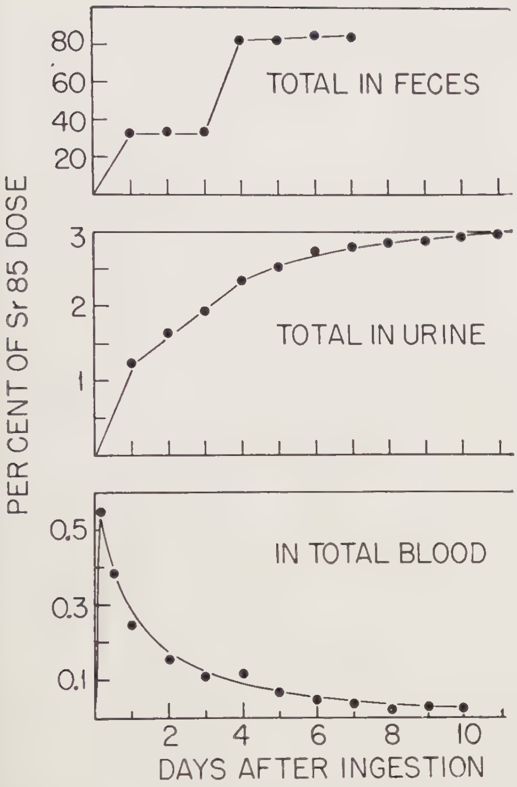

FIG. 1. Course of strontium 85 following a single ingestion (57-year-old woman with breast carcinoma and bone metastases, patient E).

conventional precipitation of calcium oxalate for beta counting of Ca 45. With this instrumentation and the relative amounts of the two radioisotopes in the samples, the Ca 45 did not contribute significantly to the scintillation counts, nor the Sr 85 to the beta counts.

Results. Single oral dosage. The course of Sr 85 following a single ingestion is illustrated in Fig. 1, which represents a study on a 57-year-old woman (E) with breast carcinoma and bone metastases. The total fecal excretion was about 86% of the ingested dose and, as shown, occurred within 4 days after ingestion. The total urinary loss was 3% with the highest excretion rate during the first day, a slower rate between 1 and 4 days, and a decreasing rate thereafter. The percentage of the dose in the total blood was calculated from blood concentrations by assuming a standard blood volume of 7.7% of the body weight. Of particular interest was that the peak concentration in the blood occurred at 4 hours or earlier after ingestion; this emphasizes the rapidity of absorption. A summary of excretions by several patients is presented in Table I. The effect of milk on increasing absorption has been discussed elsewhere(10). On the nonmilk diet the fecal excretion averaged 74% and the urinary excretion about 6%.

Daily oral dosage. Although it is theoretically possible to use single-dose data to calculate steady state levels resulting from daily ingestion, it was deemed advisable to obtain direct information. Two patients were given Sr 85 Cl_2 in water before each meal for 20 days. The over-all results are given in Table II; there is reasonable agreement between the excretion figures and those of the single dosage experiments as already presented. The time pattern is illustrated for patient F in Fig. 2. The percentage of the ingested dose retained in the body at each day was calculated by subtracting total excretion from total dose up to that day. It is of particular interest to note that the body burden of Sr 85 was

TABLE I. Fecal and Urinary Excretion of Single Dosages of Strontium 85.

Subject	Age	Sex	Diet	Condition	Feces	Urine
					% of dose	
A	64	♂	Nonmilk	Generalized lymphosarcoma	86	1.8
A	64	♂	Milk		89	.7
B	68	♂	Nonmilk	Lymphoepithelioma	55	7.7
B	68	♂	Milk		35	.5
C	58	♂	Nonmilk	Generalized lymphosarcoma	59	7.8
C	58	♂	Milk		1	3.6
D	15	♀	Nonmilk	Brain tumor, medulloblastoma	84	11
D	15	♀	Milk		17	7.8
E	57	♀	Nonmilk	Breast carcinoma	86	3.0

still increasing up to the 20th day, even though a person of this age is probably in stable strontium balance(8). Thus it appears that there is a continuing net retention of ingested radiostrontium even at a time when plateau levels have been attained in the body fluids and presumably the exchangeable sites in the skeleton are saturated. A possible explanation is that unlabeled areas of bone are probably undergoing resorption at this time so that the corresponding accretion may result in net retention of radiostrontium but not of stable strontium. It will be important to determine for how long and at what rate the body burden increases after the start of ingestion by persons with fully developed skeletons. As expected, there was only a slight loss from the body after the dosage was stopped.

The feces curve, of course, reflects the irregular passage of the stool. The urinary excretion showed an upward trend with time but it is not possible to say whether a plateau level had been reached; the sharp drop after cessation of dosing emphasizes that urinary levels are primarily an indication of the previous day's intake rather than of the body burden. The plasma level showed a steady rise and a suggestion of a slight decrease before the dosing stopped, although this may not have been real.

Of particular interest is the blood picture as a function of time after the start of daily administration. Data from several patients are shown in Fig. 3. It is noted that 2 patients with leukemia showed a low blood level and attained a plateau within 5 or 6 days. A patient with an osteolytic lesion demonstrated a steep slope during the first 6 days. Patients with ovarian carcinoma were intermediate in terms of both slope and level whereas the few points on a 9-year-old boy with cerebellar medulloblastoma indicated that a plateau had been reached by the fourth day.

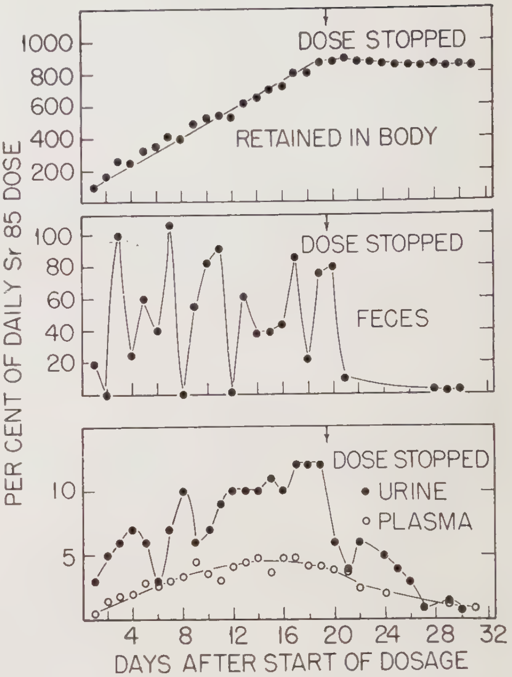

FIG. 2. Course of strontium 85 following ingestion with each meal (47-year-old woman with ovarian carcinoma, patient F).

TABLE II. Steady State Excretions and Plasma Levels of Strontium 85.

Patient	F	G
Age	47 yr	28 yr
Sex	♀	♂
Disease	Ovarian carcinoma	Granulocytic leukemia
Duration of feeding	20 days	20 days
% of dose in feces at steady state	60	60
% of dose in urine at steady state	8.8	3.2
% of daily dose in plasma at steady state	3.9	1.0

Tissue distribution. Tissue samples were available from some of the patients at autopsy at varying times after the metabolism experiments. The concentrations of Sr 85 calculated as percentage of dose per kilo of tissue are presented in Table III. Definite interpretations are difficult because of the differing experimental conditions; however, the general levels are of interest and it is clear that the behavior of the Sr 85 is at least qualitatively similar to that in animals. From the blood value of patient H at 8 hours after injection, it is inferred that there is the usual blood disappearance curve. At a few hours after injection it is noted that the blood, kidney, spleen, thyroid, bones, and bone marrow were about the same level, whereas the other tissues were much lower. At the longer

TABLE III. Tissue Distribution of Sr 85 in Human Subjects. (Values calculated as % of dose per kilo fresh wt.)

	Patient, age (years), disease			
	H	I	C	G
	73	47	58	28
	Myeloma	Ovarian cystadenocarcinoma	Generalized lymphosarcoma	Leukemia
Admin. and time schedule	Intraven., died 8 hr after dosage	Intrav., died 17 days after dosage	2 equal oral doses 32 and 19 days before death	Oral with each meal for 19 days, died 40 days after last dose
Blood	2.7	.36		
Muscle	.67	.14	.011	.15
Heart		.19		.12
Lung		.63	.35	.54
Colon	.29	.44	.021	.44
Liver	.79	.26		
Kidney	1.6	.36	.19	.24
Spleen	1.1	.29	.52	1.0
Thyroid	1.6	1.7		1.1
Adrenal		.32		
Pancreas	.70	.41		.19
Rib	2.2	8.3	.22	
Femur shaft	1.0	4.2	.29	2.3
Medullary bone				
sternum		1.3	.33	1.8
vertebra	4.0	5.4	.80	1.3
ilium	3.7	8.7		4.1

time intervals there was generally the pattern of high bone values and lower blood and soft-tissue values; for example, concentrations in the femur shaft ranged from 15 to 30 times those of muscle.

Strontium-calcium discrimination. Double tracer procedures and rationale for estimation of strontium-calcium discrimination in rats have been described(4). The same methods have been used with human subjects except that Sr 85 was employed instead of Sr 89. Terminology and equations are as follows:

Strontium-Calcium Observed Ratio (OR)

$$\mathrm{OR}_{\text{sample-precursor}} = \frac{\text{Sr/Ca of sample}}{\text{Sr/Ca of precursor}} \qquad (1)$$

Strontium-Calcium Discrimination Factor (DF)

$$\mathrm{DF}_{\text{absorptive}} = \frac{100 - \%\ \text{Sr 85 in feces}}{100 - \%\ \text{Ca 45 in feces}} \qquad (2)$$

$$\mathrm{DF}_{\text{urinary}} = \frac{\mathrm{OR}_{\text{body-diet}}}{\mathrm{DF}_{\text{absorptive}}} \qquad (3)$$

Since the major route of exposure of the human population is via milk (at least in the Western countries), the following experiments were done by giving Sr 85 and Ca 45 in milk with each meal for 5 to 20 days. A summary of results on 4 patients is presented in Table IV. The most important observation was the $\mathrm{OR}_{\text{blood-diet}}$ and the extent to which this value reflected the $\mathrm{OR}_{\text{body-diet}}$. In the interests of brevity the data are not presented, but in the first 3 patients it was noted that the $\mathrm{OR}_{\text{blood-diet}}$ on the second day of feeding and thereafter was not significantly different from average values after feeding for 15 to 20 days. Therefore it was felt that 4 to 5 days of daily feeding is adequate for this type of experiment.

With patient J complete collections of excreta were made and the $\mathrm{OR}_{\text{retained-diet}}$ was calculated by difference between dose and excretion. In agreement with previous studies on rats(4), the $\mathrm{OR}_{\text{retained-diet}}$ was slightly higher than the $\mathrm{OR}_{\text{blood-diet}}$. The $\mathrm{OR}_{\text{retained-diet}}$ should represent the ratio in the skeleton. Patient L died about one month after the last dosage and samples of rib and femur were analyzed to give $\mathrm{OR}_{\text{bone-diet}}$ values. It has been shown that the $\mathrm{OR}_{\text{bone-diet}}$ value after a single dose tends to decrease with time(2)

TABLE IV. Summary of Strontium-Calcium Discrimination in Human Subjects as Shown by Double Tracer Method.

	Patient, age, sex and disease			
	J	K	L	M
	73 yr, ♀	38 yr, ♀	25 yr, ♂	9 yr, ♂
	Primary tumor unknown	Ovarian carcinoma	Chronic granulocytic leukemia	Cerebellar medulloblastoma
Days on test	11	15	12	6
$\mathrm{OR}_{\text{blood-diet}}$	.62	.50	.52	.52
$\mathrm{OR}_{\text{retained-diet}}$	.75			
$\mathrm{OR}_{\text{bone-diet}}$			.53	
$\mathrm{OR}_{\text{urine-diet}}$	1.8	1.6	2.2	
% total excretion*				
Ca 45 in urine	1.8			
Sr 85 " "	3.1			
Ca 45 in feces	36			
Sr 85 " "	50			
$\mathrm{DF}_{\text{absorptive}}$	.78			
$\mathrm{DF}_{\text{urinary}}$	.87			
% daily dose/ml plasma†				
Ca 45	.0014	.0013	.00042	.0015
Sr 85	.00085	.00061	.00021	.0008
% daily dose in total blood†				
Ca 45	2.9	2.5	.96	1.4
Sr 85	1.8	1.2	.48	.72
% daily dose in urine/day†				
Ca 45	1.4	1.7	.10	
Sr 85	2.6	2.7	.23	

* $\frac{\text{Total in excreta}}{\text{Total ingested}} \times 100.$

† At steady state.

and this seems to fit with the values of $\mathrm{OR}_{\text{blood-diet}}$ and $\mathrm{OR}_{\text{bone-diet}}$ for this patient. The $\mathrm{DF}_{\text{absorptive}}$ and $\mathrm{DF}_{\text{urinary}}$ values of 0.78 and 0.87, respectively, are in agreement with values obtained for rats on a milk diet(4). The levels in blood and urine are in fair agreement between patients except for the low values from patient L with leukemia. It may be coincidental but it should be noted that patient G (Table II), also with leukemia, showed a lower plasma and urine level. The general plasma and urine levels were also in agreement with those reported in Table II.

Discussion. It is evident that the behavior of radiostrontium in man is not essentially different from that in animals. The major points include (a) rapid absorption from the gastrointestinal tract; (b) increased absorption and retention when milk is given simultaneously; (c) rapid removal from blood for deposition in bone and to a lesser extent excretion in the urine; (d) high retention of radiostrontium once it has been deposited in bone; and (e) preferential absorption of calcium and preferential urinary excretion of strontium. Under conditions of daily intake it is estimated that the plasma level would range from 0.0006 to 0.001% of the daily intake per ml. The daily urinary excretion is expected to range from about 1 to 10% of the daily intake, being variable between persons and reflecting primarily the previous day's ingestion.

The comparative behavior of the two elements in man is apparently similar to that in rats. There was preferential absorption of calcium from the gastrointestinal tract and preferential excretion of strontium in the urine. It can be inferred that there was little discrimination in movement between blood and bone (Table IV, patient L). This is in disagreement with the interpretations of Kulp *et al.*(2), based on single dosage studies.

It is of interest to consider the extent to which the results presented may apply to normal persons. There seems no doubt that the

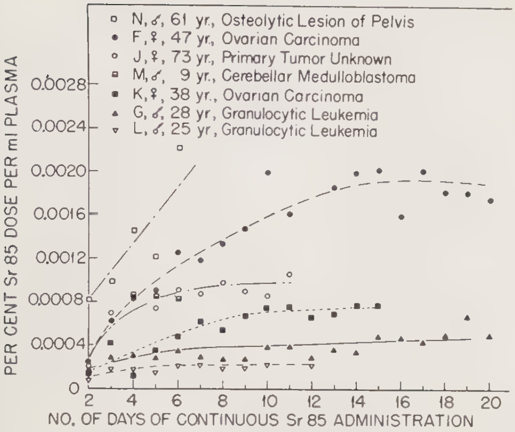

FIG. 3. Summary of plasma levels of strontium 85 following ingestion with meals over several days.

blood levels as presented in Fig. 3 indicate a dependence upon condition of the patient. The absorption values, however, are all quite similar to those reported by others(3). It is of special interest to note that similar discrimination values were obtained for patients with dissimilar conditions (leukemia *vs.* cerebellar medulloblastoma or ovarian tumor). Thus it might be reasonable to conclude that the disease condition, in these patients at least, affected the over-all behavior of calcium and strontium in the same way, if at all.

In evaluation of the discrimination that takes place against strontium in favor of calcium, we are proposing a factor of 2 for the calcium and strontium derived from dairy products, based on the data reported here. The discrimination is not known between calcium and strontium of plant origin as eaten by man. From experience with rats on a nonmilk diet, one might expect the discrimination in man on a nonmilk diet to be greater than 2, perhaps as high as 4. Some of the factors that may influence discrimination of nonmilk strontium and calcium are the presence of milk in the diet and the over-all level of dietary calcium; data are not yet available to permit estimates of the effect of such variables.

Summary. 1) These studies indicate that the behavior of radiostrontium in man is similar to that in animals. When ingested in water solution by adults, about 74% was excreted via the feces and 6% via the urine; the absorption occurred mainly within 4 hours after ingestion. Values are given for the plasma and urinary levels attained at steady state under conditions of ingestion with milk at each meal; these may be of use for monitoring purposes. 2) Two leukemia patients showed low plasma and urinary levels of radiostrontium whereas a patient with an osteolytic lesion showed a tendency towards high plasma levels. 3) When radiostrontium and radiocalcium were fed simultaneously in milk with each meal for several days there was a discrimination against strontium by a factor of about 2; this resulted almost equally from preferential absorption of calcium and preferential urinary excretion of strontium.

Note added in proof:

In recent studies with 4 patients on non-milk diets over periods up to 10 days, Dr. Daphne Papadopoulou in this laboratory observed a mean $\mathrm{OR}_{\text{blood-diet}}$ value of 0.44 with a standard deviation of ±0.05.

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Gastric Mucosal Erosion in the Rat Following Administration of the Serotonin Precursor, 5-Hydroxytryptophan. (23232)

Bernard J. Haverback and Donald F. Bogdanski (Introduced by B. B. Brodie)
Clinic of General Medicine and Experimental Therapeutics and Laboratory of Chemical Pharmacology, National Heart Institute, N.I.H., Bethesda, Maryland.

The presence of serotonin (5-hydroxytryptamine) in the gastrointestinal mucosa of the mammalian species has stimulated investigation concerning its role in the normal function and in disease of the gastrointestinal tract(1). It therefore became of interest to determine what effect administration of 5-hydroxytryptophan (5HTP), the metabolic precursor of serotonin, has on the gastrointestinal tract of the experimental animal. Previous studies have demonstrated that 5HTP is converted to serotonin *in vivo,* and measurable increases of serotonin in blood and tissue have been demonstrated within 15 minutes, reaching a maximum in about 60 minutes, and persisting for several hours after administration of 5HTP(2,3).

Methods. Seventy-five Sprague-Dawley rats weighing between 200 and 300 g were used. All substances were administered intraperitoneally except as indicated in Table I. The stomachs of the rats were examined 3 hours after administration of 5HTP.

Results. As is shown in Table I, almost all animals that received 5HTP in a dose of 300 mg/kg developed hemorrhagic gastric erosions which occurred almost exclusively in the corpus and antrum, which constitute the glandular portion of the stomach (Fig. 1). Occasionally a lesion was noted in the mucosa of the proximal duodenum. The lesions varied from 1 to 3 mm in diameter, and on microscopic examination showed necrosis of one-third of the thickness of the mucosa. Evidence of hemorrhage surrounding the necrotic material and a mild polymorphonuclear leukocytic infiltration were present.

As large doses of reserpine have been demonstrated to liberate stored serotonin from the gastrointestinal tract(4), reserpine alone was administered in an attempt to produce the gastric erosions. No erosions were found 3 hours after administration of 1 and 5 mg/kg. However, 18 hours after administration of 5 mg of reserpine per kg, hemorrhagic erosions were noted. Benditt(5) also has reported the presence of hemorrhagic erosions in the gastric mucosa of the rat following reserpine administration.

Atropine sulfate administered one hour before 5HTP was effective in preventing the erosions. As atropine had this effect, the action of 5HTP on gastric acid secretion in the rat was determined. Studies have indicated that 5HTP decreases gastric acid secretion in a Shay-rat preparation. After ligation of the pylorus, 6 rats were given 300 mg/kg of 5HTP and 5 rats were given 5 ml of isotonic saline intraperitoneally. Three hours later the mean gastric volume and acidity were 2.1 ml and 5 meq per liter in those receiving 5 HTP and 3.8 ml and 55 meq per liter in those receiving saline.

Discussion. These findings take on added significance in view of the apparent increased incidence of peptic ulceration in the malignant carcinoid syndrome where a hyperserotonemia exists. MacDonald reported an incidence of stomach and duodenal ulceration of 38% in 21 cases(6), and Manion found peptic ulceration in 4 of 6 autopsied cases.* Gastric analysis in 3 patients with malignant carcinoid syndrome did not reveal hypersecretion of acid juice. The average volume and free acidity of six 15-minute collections of basal gastric secretion in these patients were 14 ml and 9 meq per liter, respectively. Furthermore, studies in dogs have shown that when 5HTP is administered intravenously, the major effect on gastric secretion is inhibition(7). In 3 similar experiments on a dog with a simple gastric fistula and intact vagal innervation, the mean volume and free acidity of the spontaneous secretion were 20 ml and 93 meq per liter during a 40 minute period. In the interval 20 to 60 minutes after admin-

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* Personal communication.

TABLE I. Gastric Mucosal Erosions Following Administration of 5-Hydroxytryptophan and Related Compounds.

Test substance	Time of autopsy after drug admin. (hr)	No. of rats	No. with erosions	Avg No. of erosions per rat
Saline	3	10	0	0
5-Hydroxytryptophan 300 mg/kg				
Intraper.	3	16	14	4
Subcut.	3	5	5	5
Reserpine				
1 mg/kg	3	4	0	0
5	3	9	0	0
3	18	6	2	<1
5	18	4	4	3
Atropine sulfate 5 mg/kg				
5-Hydroxytryptophan 300 mg/kg	3	10	2	<1

istration of 20 mg per kg of 5HTP these values declined to 2 ml and 0 meq per liter, respectively. Although reserpine produces gastric erosions, it is not necessary to attribute these to the demonstrated stimulation of gastric secretion in dog and man(8,9).

Selye and others have demonstrated that similar erosions may be produced in the rat by a variety of drugs, traumatic injuries, burns, excessive muscular exercise, cold and other alarming stimuli(10). As serotonin occurs naturally in the stomach mucosa, it is logical to inquire whether this substance may be involved in the production of the erosions by the various stimuli.

Summary. 5-Hydroxytryptophan administered to rats in a dose of 300 mg/kg produced hemorrhagic mucosal erosions in the glandular portion of the stomach. When atropine sulfate was injected prior to 5-hydroxytryptophan administration, the lesions were decreased in number or their occurrence prevented. Administration of reserpine also produced similar lesions which were delayed in appearance.

The authors wish to thank Dr. Alan Rabson for his cooperation.

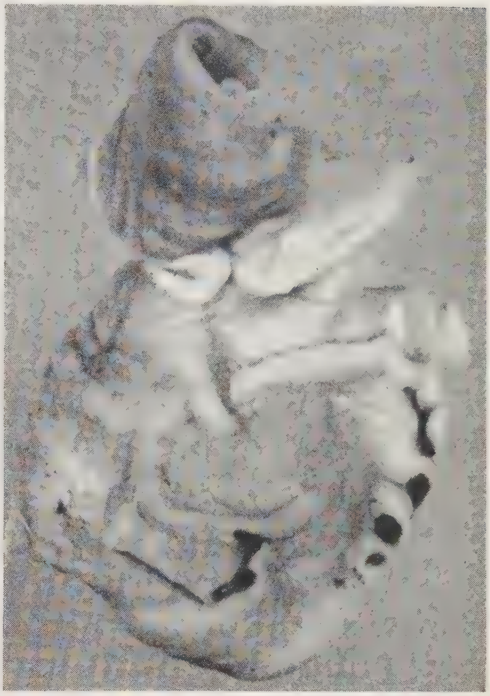

FIG. 1. Hemorrhagic gastric erosions occurring in glandular portion of the stomach of the rat 3 hr after administration of 5-Hydroxytryptophan in a dose of 300 mg/kg.

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Human Endogenous Plasma Lipemia Clearing and Lipolytic Activity After Tricalcium Phosphate Adsorption. (23233)

H. Engelberg*
Division of Laboratories, Cedars of Lebanon Hospital, Los Angeles, Calif.

Nilsson and Wenckert(1) demonstrated that heparin was adsorbed to known prothrombin adsorbents. Subsequently Nikkila and Pesola(2) reported that post-heparin clearing factor was adsorbed on tricalcium phosphate gel. Human endogenous plasma lipemia clearing factor has been found in some subjects(3). Further work(4) demonstrated its lipolytic nature and inhibition characteristics which were similar to the post-heparin enzyme (lipoprotein lipase) and different from those of pancreatic lipase. In the present study the effect of tricalcium phosphate adsorption on clearing and lipolytic activity of human post-heparin plasma, pancreatic lipase and endogenous plasma lipolytic activity has been investigated. The results add further evidence of the identity between the endogenous plasma lipolytic enzyme and the post-heparin factor, and support the conclusion that heparin is normally a component part of lipoprotein lipase essential to or stabilizing its activity.

Methods. Tricalcium phosphate gel was prepared as described by Quick(5). (This must be completely salt-free or clearing factor adsorption does not occur.) Blood was obtained using chilled syringes and oxalated tubes, and the plasma separated in refrigerated centrifuge. (Citrate interferes with adsorption of heparin on the gel, and heparin was not used as anticoagulant because of the remote possibility of slight *in vitro* clearing factor formation.) One ml of plasma was added to 0.1 ml of gel and the mixture allowed to stand for 30 minutes at 4°C with frequent shaking. In studies of clearing activity, one ml of the original plasma or one ml of the supernatant liquid after adsorption were mixed with .4 ml of a .25% suspension of Ediol. Optical density changes were followed for one hour at 25°C using a Coleman Jr. spectrophotometer at 540 mμ. In the investigations of lipolysis a series of tubes containing one ml aliquots of plasma before and after adsorption were incubated at 37°C for 15, 30 and 60 minutes with .2 ml of human low density serum lipoproteins† added to each tube. It had been previously determined that at this level of added lipoproteins the amount of fat substrate does not affect the rate of lipolytic reaction. Unesterified fatty acids were determined using the method of Borgstrom(6) which we found to have a maximum deviation from the mean of .05 Meq/l. Calculation of rate of release of unesterified fatty acids per hour was based upon the maximum increment of fatty acids found in any time period. This assumption is justified since in this *in vitro* system the end products of the reaction (glycerol and free fatty acids) are not removed and these inhibit further lipolysis(7). Esterified cholesterol was also measured before and after one hour incubation since we have found that occasionally an increase in cholesterol ester occurs which, if undetermined, might mask a moderate degree of fatty acid release. Human pancreatic lipase was obtained from fresh autopsy material.‡

Results. Table I shows the effect of tricalcium phosphate gel adsorption upon lipemia clearing and lipolytic activity of post-heparin plasma and pancreatic lipase. There is a marked reduction of activity in the former but no decrease or perhaps an increase in the fat splitting action of the pancreatic lipase. Occasionally activity of the post-heparin factor is completely removed by the gel.

Table II presents the results of optical den-

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* Supported by U.S.P.H.S. grant.

† Ultracentrifugally separated and supplied by the Inst. of Medical Physics through the courtesy of Mr. D. Spector. The lipoproteins represent a 5-fold concentration of predominantly Standard Sf 12-400 lipoproteins in their original sera.

‡ Supplied through cooperation of Dr. N. Friedman, and extracted by the Southern California Gland Co.

TABLE I. Effect of Tricalcium Phosphate Adsorption on Lipemia Clearing and Lipolytic Activity of Plasma Plus Human Pancreatic Lipase, and of Human Post-Heparin Plasma.

Preparation	$CA_3(PO_4)_2$ Adsorption	Incubation time at 25°C Optical density readings* 0	10′	20′	30′	60′
Pre-heparin plasma + human pan. lipase	B	.37	.36	.35	.35	.34
	A	.40	.38	.37	.37	.34
Post-heparin plasma (100 mg I.V.)	B	.46	.43	.39	.36	.28
	A	.49	.49	.49	.49	.48
		Incubation time at 37°C				
		Unesterified fatty acids† 0	15′	30′	60′	Rate of release
Pre-heparin plasma + human pan. lipase	B	1.2	1.3	1.3	1.4	.4/hr
	A	1.0	1.5	1.5	1.3	2.0
Post-heparin plasma (100 mg I.V.)	B	2.0	3.1	3.9	4.2	4.4
	A	1.5	1.7	1.8	1.8	.8

* Read in Coleman Jr. Spectrophotometer at 540 mμ.

† Values in milliequivalents/liter.

B = before.

A = after.

sity studies before and after adsorption on the gel in patients who had endogenous lipemia clearing factor in the plasma. With the exception of subject E.M. the post-adsorptive plasma had less or no clearing activity. The plasma of subject E.M. was repeatedly tested and in no instance did gel adsorption affect activity, although it was inhibited by sodium taurocholate but not by protamine sulfate. No release of free fatty acid could be demonstrated when his plasma was incubated with human lipoproteins so that this probably represents an instance of non-lipolytic clearing. In several instances in other subjects (M.A., J.S., C.S., A.K.) the clearing activity which was adsorbed in the phosphate gel was inhibited by 5% sodium taurocholate, TEPP, molar NaCl and protamine, which inhibit post-heparin factor but do not affect pancreatic lipase activity(4).

Studies in 14 individuals of the release of unesterified fatty acids before and after plasma adsorption on tricalcium phosphate are shown in Table III. In 3 of the 14 subjects (M.E., E.H. and B.L.) endogenous lipemia clearing activity had been found on previous occasions, but the others had not been tested before. Four of these showed an increase in unesterified fatty acids, and in 2 (S.B. and W.N.) the increase in esterified cholesterol masked a release of the fatty acids. In 8 of the 9 subjects in whom lipolysis occurred, the activity was either completely or almost completely removed by adsorption with the gel. In one, M.E., although the rate of release of fatty acids was apparently unchanged, there is evidence of a more rapid inactivation of the lipolytic activity upon incubation. The measurements of cholesterol ester reveal that in every instance a slight decrease was found after adsorption, and that less esterification occurred in the adsorbed plasma.

TABLE II. Effect of Tri-Calcium Phosphate Adsorption on Human Endogenous Plasma Lipemia Clearing Activity. Optical Density Readings at 540 mμ. Tubes Contained 1 ml Plasma Plus 0.4 ml .25% Ediol.

Patient Sex age	$Ca_3(PO_4)_2$ Adsorption	Incubation time at 25°C 0	10′	30′	60′
L.D.	B	.54	.46	.30	.40
♂ 44	A	.36	.37	.38	.38
L.R.	B	.49	.46	.46	.50
♀ 50	A	.49	.49	.51	.51
I.F.	B	.47	.50	.50	.46
♂ 64	A	.49	.51	.50	.49
T.H.	B	.44	.43	.39	.38
♂ 49	A	.47	.47	.46	.46
E.M.	B	.36	.33	.31	.29
♂ 62	A	.41	.39	.36	.33
D.H.	B	.35	.34	.33	.33
♀ 42	A	.32	.32	.31	.31
M.A.	B	.40	.36	.32	.30
♂ 70	A	.56	.54	.52	.50
	I.M. NaCl	.25	.26	.28	.28
	TEPP 1×10^{-1}	.41	.40	.40	.40
G.S.	B	.42	.40	.40	.37
♂ 65	A	.41	.41	.41	.40
	5% Sod. Taur.	.41	.41	.41	.41
J.S.	B	.65	.64	.63	.63
♂ 32	A	.66	.66	.66	.66
	5% Sod. Taur.	.58	.58	.58	.575
H.M.	B	.40	.39	.37	.37
♀ 35	A	.37	.38	.37	.36
H.K.	B	.29	.27	.24	.21
♂ 63	A	.35	.35	.34	.31
	TEPP 1×10^{-1}	.31	.31	.31	.30
	5% Protamine	.30	.30	.29	.26

B = before.

A = after.

TABLE III. Effect of Tri-Calcium Phosphate Adsorption on Endogenous Lipolytic Activity of Oxalated Human Fasting Plasma. Tubes Contained 1 ml Plasma Plus 0.2 ml Human Low Density Serum Lipoproteins.

		Incubation time at 37°C						
		Unesterified fatty acids in mEq/l					Chol. esters	
Patient Sex age	$Ca_3(PO_4)_2$ Adsorption	0	15′	30′	60′	Rate of release/hr	0	60′
M.E.	B	.2	.4	.6	.6	.8	235	263
♂ 36	A	.2	.4	.5	.5	.8	230	233
E.H.	B	.2	.4	1.7	1.9	3.0	185	185
♀ 41	A	.2	.4	.6	.6	.8	180	180
A.N.	B	.4	.4	.5	.6	.2	224	224
♂ 45	A	.4	.4	.4	.4	.0	197	196
A.K.	B	.3	.3	.3	.5	.2	198	200
♀ 50	A	.3	.3	.3	.3	.0	183	183
A.B.	B	.4	.5	.6	.6	.4	271	274
♀ 42	A	.4	.4	.4	.5	.1	214	213
D.S.	B	.3	.3	.3	.3	.0	227	227
♀ 42	A	.3	.3	.3	.3	.0	224	224
P.S.	B	.2	.2	.2	.2	.0	156	156
♀ 44	A	.2	.2	.2	.2	.0	142	142
S.B.	B	.3	.3	.3	.3	.12	185	193
♂ 71	A	.3	.3	.3	.3	.04	176	179
D.Y.	B	.7	.7	.7	.7	.0	166	166
♀ 47	A	.6	.6	.6	.6	.0	161	161
J.Z.	B	.3	.3	.3	.3	.0	168	169
♂ 26	A	.3	.3	.3	.3	.0	147	147
R.F.	B	.5	.5	.5	.5	.0	212	212
♀ 62	A	.5	.5	.5	.5	.0	176	176
S.H.	B	.5	.8	.9	1.0	1.2	220	249
♂ 47	A	.4	.5	.6	.7	.4	189	202
B.L.	B	.6	.9	.9	.9	1.2	184	190
♂ 32	A	.6	.5	.5	.5	.0	176	178
W.N.	B	.7	.7	.7	.7	.18	230	242
♂ 66	A	.7	.7	.7	.7	.0	226	226

B = before.
A = after.

Discussion. The finding that tricalcium phosphate gel adsorbs post-heparin factor but does not affect pancreatic lipase activity affords another useful method of distinguishing between these two lipolytic enzymes. The results of the gel adsorption of the endogenous plasma clearing and lipolytic activity thus strongly support previous studies(4) indicating its identity with heparin lipoprotein lipase.

We found that after tricalcium phosphate adsorption endogenous heparin cannot be demonstrated in the supernatant plasma with octylamine precipitation methods(1). However, markedly decreased clearing and lipolytic activity may remain in some cases. This suggests that minor or unstable lipoprotein lipase activity may exist without heparin. Thus Robinson(8) found that removal of heparin from post-heparin plasma by a resin does not destroy the enzyme system but markedly reduces its stability. Korn(9) showed that a bacterial heparinase produced a partial (approximately 60%) inactivation of tissue lipoprotein lipase activity. His results indicated that heparin was an integral part of the molecule but that destruction of the former did not totally prevent lipolysis of lipoproteins by the remaining part of the enzyme. The effect of tricalcium phosphate adsorption is in accord with these findings.

The post-adsorptive plasma had less esterified cholesterol, and showed a diminution in the further esterification of cholesterol upon incubation, than the pre-adsorbed plasma. Although the meaning of this is unclear at this time, the apparent removal of some cholesterol esterase activity by tricalcium phosphate gel, a known heparin and prothrombin adsorbent, raises the possibility of some relationship between these substances. Unfortunately determinations of total cholesterol before and after adsorption were not done.

Summary. Tricalcium phosphate gel removes all or the major portion of post-heparin clearing factor activity from plasma but does not decrease the activity of human pancreatic lipase. It is therefore a useful agent in identifying heparin lipoprotein lipase. Endogenous plasma lipolytic activity is completely or nearly completely removed after gel adsorption in almost all subjects in whom it is present, affording further evidence of its similarity to or identity with post-heparin factor. Plasma cholesterol ester and cholesterol esterase is also apparently decreased in the post-adsorptive sample.

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Paper Electrophoresis of Animal Hemoglobins. (23234)

Gerald P. Rodnan* and Franklin G. Ebaugh, Jr.†
(Introduced by Jessica H. Lewis)
Department of Medicine, University of Pittsburgh and Nat. Inst. of Arthritis and Metabolic Diseases, N.I.H.

The discovery of an abnormal hemoglobin in sickle cell disease has stimulated intensive electrophoretic study of human hemoglobin (1-4). An interest in comparative aspects of erythrocyte turnover(5) led us to undertake electrophoretic characterization of hemoglobins of a variety of animal species. A wide spectrum of migration patterns was found, with some species demonstrating as many as 3 electrophoretically separable hemoglobins.

Materials and methods. Blood was obtained from active adult animals (and 3 calves) (Table I) by cardiac or venipuncture and either oxalate or heparin used as anticoagulant. (Penguin blood was taken from an animal severely ill with aspergillosis.) Human blood was drawn from several healthy adult males, from patients with sickle cell disease(6), and from an individual with hemoglobin C disease(7). Blood cells were washed 3 times with 0.9% NaCl, lysed with 2-3 volumes of distilled water, and centrifuged to separate out particulate (stromal) elements. 0.005-.020 ml samples of hemoglobin solutions (stored frozen until electrophoresis) were stripped on filter paper and placed in a Spinco electrophoresis cell(8) containing barbital buffer of .01 ionic strength, pH 9.2. Voltage was maintained constant at 270 volts for a 5 hour period of electrophoresis. Under these conditions the average current across each 3 cm wide paper strip was .5 m amp. Human A hemoglobin, and usually S and C hemoglobins as well, were run simultaneously in the same cell with animal hemoglobins. Human A hemoglobin

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* Public Health Service Research Fellow of Nat. Heart Inst.

† Present Address: Hitchcock Clinic and Dartmouth Medical School, Hanover, N. H.

TABLE I. Animal hemoglobins subjected to electrophoresis. Figures in parentheses indicate number of individuals tested.

Mammals
  Man
  Chimpanzee (*Pantroglodytes*) (1)
  Rhesus (*Macaca mulatta*) (4)
  Macaque (" *syrichta*) (1)
  Llama (*Lama glama*) (1)
  Horse (draft) (5)
  Cow (Hereford) (7)
  Calf (") (3)
  Pig (Hampshire, Berkshire) (4)
  Sheep (Hampshire) (6)
  Goat (brush) (2)
  Dog (mongrel) (14)
  Cat (") (7)
  Mouse (white, random bred) (5)
  Rat (Sprague-Dawley, ") (6)
  Guinea pig (Hartley-Beltsville hybrid) (5)
  Rabbit (New Zealand White) (7)
  Hamster (Golden Syrian) (5)
Birds
  Chicken (N. H., mongrel Leghorn) (11)
  Pigeon (white Carneaux) (6)
  Duck (white Pekin) (9)
  Robin (*Turdus migratoria*) (1)
  Penguin (*Aptenodytes forsteri*) (1)
Reptiles
  Turtles:
    Terrapene carolina carolina (5)
    Pseudyms floridana (2)
    " *rubriventris* (1)
  Black Snake (*Elaphe obsoleta*) (1)
  Water " (*Natrix taxispilota*) (1)
Amphibia
  Toad (*Bufo marinus*) (2)
  Frog (*Rana pipiens*) (7)
Fish
  Carp (*Cyprinus carpio*) (1)

migrated approximately 6 cm in 5 hours, with a variation of as much as 0.5 cm between 2 strips in the same cell. Conclusions in regard to relative mobility were based on multiple runs in which consistent significant differences were observed. Blood from one turtle (*Terrapene carolina*) was incubated *in vitro* with $Na_2Cr^{51}O_4$, which has been found to bind firmly with both human and animal hemoglobin(5). Hemoglobin thus labelled was submitted to electrophoresis and separated into 2 components as described below. The paper strip was run through a densitometer[‡] then cut transversely into 1-3 cm pieces bearing varying amounts of pigment, which were placed in counting vials and the emissions of chromium^{51} scanned in a well-type scintillation counter.

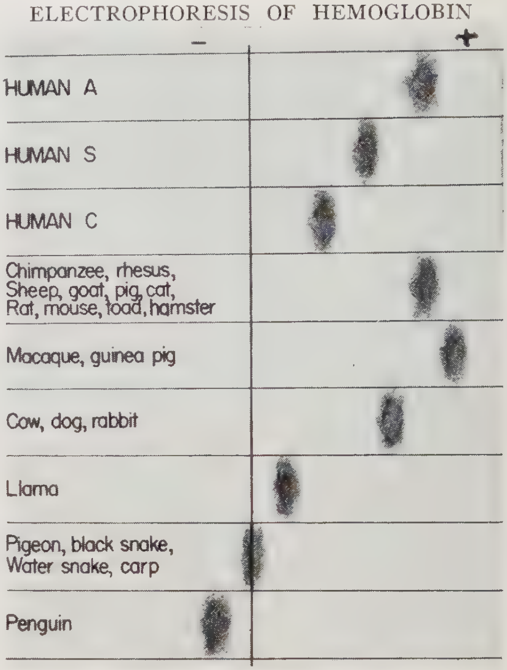
ELECTROPHORESIS OF HEMOGLOBIN
−
+
HUMAN A
HUMAN S
HUMAN C
Chimpanzee, rhesus, Sheep, goat, pig, cat, Rat, mouse, toad, hamster
Macaque, guinea pig
Cow, dog, rabbit
Llama
Pigeon, black snake, Water snake, carp
Penguin

FIG. 1. Diagrammatic representation of electrophoretic patterns of various human hemoglobins and of animal species demonstrating single-component hemoglobins.

Results. Mammalian hemoglobins. The pattern of mammalian hemoglobins displaying single electrophoretic components is shown in Fig. 1. Those found to have anodic mobility similar to human A hemoglobin included chimpanzee, rhesus monkey, pig, goat, cat, mouse, rat, hamster, and 4 of 6 sheep. Hemoglobin of guinea pigs and a macaque possessed greater mobility, while that from llama, cow, dog, and rabbit migrated less rapidly than human A hemoglobin.

A number of species disclosed 2-component patterns (Fig. 2). Hemoglobins of 5 draft horses gave identical separations of double bands slightly faster than human A. While each of 7 adult Hereford steer presented single hemoglobins moving more slowly than A (Fig. 1), hemoglobins from 2 of 3 Hereford calves (estimated age 2-6 months) contained 2 components, one equal in anodic mobility to that of adult steer hemoglobin, the other slower (Fig. 2). The third calf

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[‡] Spinco Analytrol, Specialized Instruments Corp., Belmont, Calif.

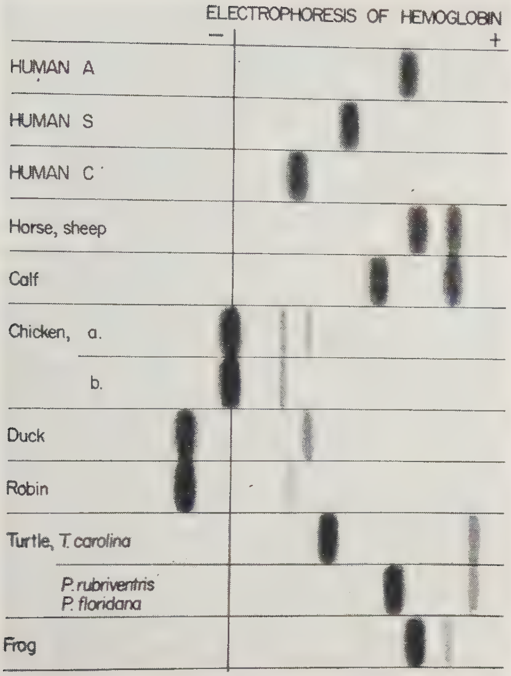

FIG. 2. Diagrammatic representation of electrophoretic patterns of various human hemoglobins and of animal species demonstrating multiple component hemoglobins.

hemoglobin had the pattern of adult steer. Two of 6 Hampshire sheep hemoglobins tested each showed 2 components, one with mobility of A hemoglobin, the other faster (Fig. 1, 2).

Avian hemoglobins. Hemoglobin from each of 6 New Hampshire chickens separated into 3 components; the major band (approximately 70%) migrated little if any distance from point of origin, while 2 faint lesser bands moved toward the anode (Fig. 2, a). One of 5 mongrel Leghorns also displayed 3 hemoglobin components; the others had but 2, lacking the more rapid of the components moving toward the anode (Fig. 2, b). Hemoglobin from each of 9 ducks separated into a major component (85%) migrating toward the cathode and a lesser component (15%) directed toward the anode. Robin hemoglobin also proved separable into a major positively charged component and a lesser negatively charged moiety (Fig. 2). Penguin and pigeon hemoglobins travelled as single bands, the former moving slowly to the negative pole, the latter displaying no significant deviation from the point of origin (Fig. 1).

Reptile hemoglobins. Hemoglobins of each of 3 species of turtle—*Terrapene carolina, Pseudyms floridana,* and *Pseudyms rubriventris*—possessed 2 widely separated bands migrating toward the positive electrode, one component somewhat more rapidly moving than human A hemoglobin, the other more slowly moving (Fig. 2). Hemoglobin of one turtle (*Terrapene carolina*), labelled *in vitro* with radioactive chromium^{51} before electrophoresis, was found to have 2 peaks of radioactivity which coincided with visible pigment peaks. Specific activities of the 2 hemoglobin components were nearly equal (Fig. 3). The black snake and water snake were found to have single component hemoglobins which moved but little from the origin point (Fig. 1).

Amphibian and piscian hemoglobins. Each of 2 toads presented single hemoglobins with mobility and direction similar to that of human A (Fig. 1). In 7 frogs, there was a

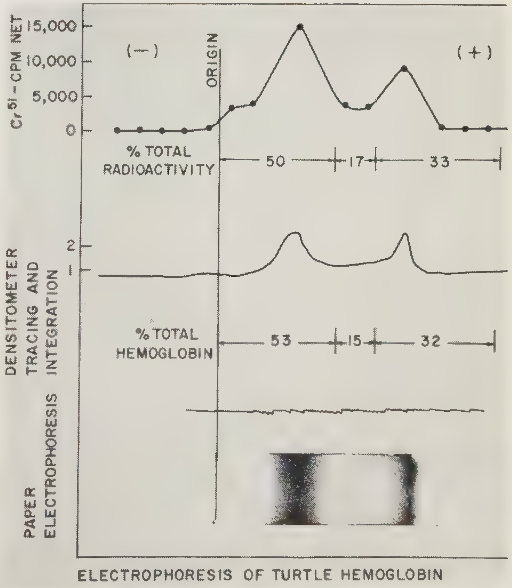

FIG. 3. Paper electrophoresis pattern, densitometer tracing, and radioactivity assay of turtle hemoglobin (*T. carolina*) labelled with radioactive chromium *in vitro*. The 2 electrophoretically separable components appear to have been labelled proportionately as indicated by comparable specific radioactivity of each peak. (Specific radioactivity = % total radioactivity/% total hemoglobin.)

heavy band moving faster than A hemoglobin, closely preceded by a faint spot suggestive of a second faster-moving component. When samples were run for periods of 8 hours, 2 components were more clearly separable (Fig. 2). In the case of carp hemoglobin there was rather wide diffusion about the line of origin, but no evidence of multiple bands (Fig. 1).

Discussion. Landsteiner and co-workers (9) determined electrophoretic mobilities of hemoglobins from several animal species, using Tiselius apparatus. The present study confirms their finding of similar mobilities in hemoglobin of the rabbit, dog, and sheep, somewhat greater mobility of horse and guinea pig hemoglobin, and slower migration of chicken hemoglobin. An ever-increasing number of reports concerned with the electrophoresis of animal hemoglobins has appeared in recent years(10-18).

Several groups have now reported the finding of 2 components in the electrophoresis of chicken hemoglobin(10-12). In the present study separation of the hemoglobin from all 6 New Hampshire birds and one Leghorn into 3 components and from 4 other Leghorns into 2 components indicates species differences in hemoglobin composition in this fowl. Our finding of double-component hemoglobins in the duck and robin and of single-component hemoglobin in the pigeon is confirmed by Dunlap, Johnson, and Farner(12). These workers studied the blood of 21 species of birds and, with the exception of the pigeon, found 2 hemoglobin components in all.

In the case of the Hereford cow, it would appear likely that temporary persistence of a fetal form of hemoglobin(19) accounts for the finding of double hemoglobin components in the calf and single components in adult animals. Study of the oxygen dissociation of fetal and maternal blood has demonstrated a developmental adult change in hemoglobin not only in the cow, but in a variety of other species including the goat, rabbit, sheep, chicken, frog, and turtle(20). Cabannes and Serain(14) found 15 of 80 bovine hemoglobins to possess 2 components and, excluding genetically transmitted variations, suggested that the more rapid of these represented an abnormal hemoglobin associated with illness in the donor. It would appear possible to us that these 2-component bovine hemoglobins represented instances of persisting fetal together with adult type hemoglobin.

In some of those species in which there are individual differences in the composition of hemoglobin from adult animals, there is evidence which indicates that the variations in hemoglobin types are genetically determined. Harris and Warren and their co-workers(18) observed a transmission of hemoglobin types in Scottish blackface sheep consistent with the view that these are determined by 2 alleles each responsible for the formation of one kind of hemoglobin. Similar variations in the hemoglobins of many hundred cattle, sheep, goats, horses, and mules were reported by Cabannes and Serain(16) who, however, did not present genetic studies in their animals. From the studies of hemoglobin patterns in inbred strains Ranney and Glueck-sohn-Waelsch(17) propose genetically based differences in mouse hemoglobin. Blood smears from each of the types observed disclosed no morphologic differences of the erythrocytes.

Svedberg and Hedenius(21) suggested that the formation of dimers and trimers might account for individual hemoglobins with 2 or more sedimentation constants in some species of reptiles and amphibians, and it might be argued that electrophoretic heterogeneity of turtle and frog hemoglobin could be due to such double molecules. Such conjugates were not observed in other classes of vertebrates (including birds) and it would appear more likely to us that differing molecules rather than dimer formation account for the finding of multiple hemoglobin components.

In those species in which 2 component hemoglobins are of regular occurrence in adult animals (horse, birds, turtles, frog) it would seem likely that both components represent normal forms of hemoglobin. Perhaps in some there is continued production of a fetal type hemoglobin into adult life. Convincing evidence linking the presence of multiple hemoglobins with hematologic disease in animals is (still) lacking.

Summary. Differences have been found

both in the number of components electrophoretically separable from individual animal hemoglobins and in the mobility of these components relative to human A, S, and C hemoglobins. Under the conditions employed, with the exception of the horse, 2 of 3 calves, and 2 of 6 sheep, all of which possessed 2 components, the (adult) mammalian hemoglobins studied were found to have single components. Some displayed anodic mobility similar to that of human A hemoglobin (chimpanzee, rhesus monkey, pig, sheep, goat, cat, mouse, rat, hamster), others had greater mobility (macaque, guinea pig), and some possessed lesser mobility (llama, cow, dog, rabbit). In the Hereford steer, it would appear that temporary persistence of fetal hemoglobin accounts for the finding of 2-component hemoglobin in the calf and single-component hemoglobin in adults. Hemoglobin from several New Hampshire chicken and one Leghorn was found to have 3 electrophoretic components; that from other Leghorns, ducks, and a robin showed 2 components. Penguin and pigeon hemoglobin had single components. Hemoglobin prepared from each of 3 species of turtles possessed 2 widely separated bands. Black snake, water snake, and toad had one-component hemoglobins. Frog hemoglobin gave a 2-component pattern. With few exceptions the number of individual hemoglobins tested in any species has to date been small. It is likely that the study of larger numbers of animals will uncover additional examples of heritable variations in hemoglobin types.

We thank Dr. Harvey Itano for his help in preparation of this report.

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Effect of Calcium Pantothenate on Isoniazid Toxicity in the Guinea Pig.*† (23235)

R. W. Manthei (Introduced by J. M. Coon)
Department of Pharmacology, Jefferson Medical College, Philadelphia.

The treatment of tuberculosis with high doses of isoniazid (isonicotinic acid hydrazide) is frequently limited by development of peripheral neuropathy in the patient(1). Central stimulation with or without convulsions results from acute toxic doses of isoniazid in man as well as in animals but chronic administration of low doses does not produce significant toxic side reactions(2). Peripheral neuropathy characterized by muscle spasticity and abnormal gait has been produced in rats maintained for several months on a diet containing 0.25% isoniazid(3). In the present study repeated administration of subconvulsive doses of isoniazid produced in the guinea pig a toxic reaction which in certain aspects resembles the peripheral neuropathy reported in man. Daily administration of calcium pantothenate to these animals prevented development of this reaction.

Materials and methods. Guinea pigs of either sex and of mixed breed weighing 100-200 g were housed in individual cages and maintained on Purina rabbit chow checkers plus cabbage. Animals were weighed each morning and isoniazid or a combination of isoniazid and ethanol was administered in 0.9% saline by intraperitoneal injection 3 times a week. Experiments were conducted for 4 to 6 weeks after which the animals were sacrificed and examined for gross pathological changes. Except for local necrosis at the injection site in a few of the animals receiving alcohol, no such changes were observed.

Results. Within one hour after an injection of 50-75 mg/kg of isoniazid the guinea pig becomes restless, easily startled and excitable and after another one or 2 hours behaves normally. However, in about 25% of the animals which have received 3 to 5 injections of isoniazid on alternate days, the period of hyperexcitability is followed by muscle tremors but overt convulsions do not develop. By 2 hours after injection the animal squeals frequently and makes obvious efforts to remain off his feet. Finally, coordinated control of the hind limb musculature is lost and the guinea pig lies on his side. His reflexes are hyperactive and an increased sensitivity to pain in the hind limbs is apparent. Merely touching any hind limb joint during this period causes the animal to withdraw his leg violently. This hyperesthesia persists for an hour or more. Atonia, initially evident only in the hind limbs, may progress forward until a flaccid paralysis of all of the body musculature is present. (This total flaccid paralysis was used as the criterion for the response throughout the remainder of the study.) Respiratory muscles, however, are not affected since respiration continued normally except in a few cases in which fluid accumulated in the respiratory tract after several hours of paralysis. The majority of animals appear normal within 5 hours after injection of the isoniazid. The guinea pigs usually lose weight on the day of injection but regain their weight on the following day and are able to maintain a normal growth curve over the experimental period. The majority of guinea pigs did not react positively to every subsequent injection of isoniazid but to only every third or fourth test with this drug when given on alternate days.

Injection of a 15% solution of ethanol in a dose of 1.2 g/kg together with the isoniazid produced a significant increase ($p(\chi^2) < 0.001$) in incidence of response in the guinea pigs (Table I). This dose of alcohol alone did not produce any observable reaction when injected 3 times a week into control animals. However, when given in combination with

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† Aided in part by medical research grant from Nat. Tuberculosis Assn., the Am. Trudeau Soc. and research grant from Nat. Inst. of Neurol. Dis. and Blindness, P.H.S.

TABLE I. Toxic Response to IP Injection of Isoniazid and Alcohol in Guinea Pigs.

Drug and dose	No. animals responding/No. animals tested	% animals responding
Isoniazid 50-75 mg/kg	10/39	25.7
Idem + alcohol 1.2 g/kg	22/27	81.5
Alcohol 1.2 g/kg	0/6	0.

isoniazid, not only did alcohol cause more animals to respond but the responses appeared earlier in the sequence of injections and with greater regularity thereafter. In many cases a response to the combination of isoniazid and alcohol was seen on initial injection of these drugs. This never occurred with isoniazid alone. Some animals responded to each injection of isoniazid and alcohol while others did not respond to the first injection given at the beginning of the week but only to the second and third injections.

The symptomatology of the response to the combination of isoniazid and alcohol was in every way similar to that seen after isoniazid alone. An increase in the incidence of response to isoniazid was also obtained in a limited number of animals in which the alcohol was given orally. Other agents given along with isoniazid but which did not significantly increase the incidence of toxic response included sodium salicylate, cortisone and acetaldehyde.

Since the response to the combination of isoniazid and alcohol was more consistent than that to isoniazid alone, these 2 drugs were used to test ability of certain vitamins to protect against the toxic response. These vitamins were injected daily in guinea pigs that had previously given 2 consecutive responses to isoniazid and alcohol. The vitamins were always given some 18 hours prior to the challenging doses of isoniazid and alcohol in order to decrease likelihood of protection being achieved by a direct chemical interaction in the bloodstream.

While control animals showed continuing positive responses (total flaccid paralysis) to test doses of isoniazid and alcohol, animals treated with calcium pantothenate no longer responded (Table II). A single injection of 20 mg/kg of the vitamin blocked the reaction in most cases on the following test day. With 10 mg/kg of calcium pantothenate several daily injections of the vitamin were required to prevent the reaction from appearing in all of the guinea pigs. However, in these animals there was a noticeable diminution of intensity of response even after the first injection of the vitamin. All animals on pantothenate therapy showed the initial stages of excitement after receiving the isoniazid and alcohol, but the spasticity, joint sensitivity and flaccidity seen previously did not appear. The decrease in incidence of total paralysis in pantothenate treated animals as compared to controls is statistically significant ($p(\chi^2) < 0.01$). When the pantothenate therapy was discontinued, responses to the isoniazid and alcohol combination again appeared after 3 to 5 injections. In a separate experiment, 2 guinea pigs giving repeated responses to only isoniazid were similarly protected by pantothenate.

Calcium ion equivalent to that present in the calcium pantothenate was administered as the gluconate to 4 guinea pigs responding to isoniazid and alcohol. These animals continued to respond to succeeding challenges of isoniazid and alcohol until they ultimately died during a period of prolonged paralysis.

Daily administration of 20 mg/kg of pyridoxal HCl in a similar series of experiments was without effect on either incidence or duration of responses to isoniazid plus alcohol. Pyridoxal administered together with or shortly after the isoniazid-alcohol combination, however, noticeably decreased severity of the responses.

Discussion. The initial hyperreflexia, the

TABLE II. Antagonism of Toxic Action of 75 mg/kg Isoniazid plus 1.2 g/kg Ethanol (IP) in Guinea Pig.*

Treatment and mg/kg	Response ratios on alternate days					
	1	2	3	4	5	6
Control	6/6	4/6	5/6	6/6	5/6	6/6
Ca pantothenate 10	8/9	1/9	2/9	1/9	0/9	0/9
Idem 20	1/6	0/6	0/6	0/6	0/6	0/6
Pyridoxal HCl 20	4/4	4/4	3/4	4/4	4/4	4/4
Ca gluconate 18	4/4	4/4	4/4	3/3	3/3	3/3

* Control and treated animals selected only after they had given 2 consecutive responses to isoniazid-ethanol.

hyperesthesia particularly evident in all the hind limb joints, and to some extent the muscle flaccidity present in these guinea pigs were all indicative of peripheral neuropathy. However, the extensive flaccid paralysis noted as well as the transient nature of the response are in general not characteristic of drug-induced polyneuritis. Hence, the toxic response from isoniazid observed in these guinea pigs differs in these aspects from the peripheral neuropathy produced in patients by isoniazid therapy.

The potentiation of toxic response from isoniazid by alcohol in these studies may have some significance in therapeutics. Oestreicher *et al.*(4) noted that 7 out of the 8 cases of peripheral neuropathy found in a series of patients on isoniazid therapy occurred in chronic alcoholics. Although isoniazid may merely have activated a sub-acute polyneuritis in such patients, it is possible that their alcohol intake may have directly contributed to the increased incidence of toxic response. Since, in the present studies, orally administered alcohol gave essentially the same reaction as injected alcohol, the increase in toxicity noted with alcohol probably did not result from local irritation by the injected alcohol. Negative results with acetaldehyde indicated that alteration in the metabolism of alcohol was also probably not an essential factor in causing the response. Since neither sodium salicylate nor cortisone altered the incidence of response, it is not likely that the response was mediated through adrenal stimulation by isoniazid.

The peripheral neuropathy seen in isoniazid treated patients has been associated with alteration in vit. B_6 activity by the drug(1). Pyridoxine HCl has been shown to relieve isoniazid induced polyneuritis in patients(5). Concomitant administration of pyridoxal HCl and isoniazid in the present studies did noticeably decrease the severity of response in the guinea pigs. However, when the pyridoxal was given some 18 hours prior to the isoniazid, no protection was noted. Thus it is likely that the protection noted in the former experiments was dependent on some effect of the high blood level of pyridoxal present. This suggests that the isoniazid toxicity observed in these studies is not directly a result of vit. B_6 inactivation in the tissues.

A peripheral neuropathy similar to that resulting from isoniazid therapy in patients has been associated with a pantothenate deficiency in man(6). Pantothenate deficiency is known to cause a peripheral neuropathy characterized by abnormal gait in swine(7). However, since a sustained peripheral neuropathy was not seen in the present studies, it is not likely that the response to isoniazid in the guinea pig is dependent on a drug induced tissue deficiency of pantothenic acid. Since a high concentration of pantothenate blocked the transient response to isoniazid in these guinea pigs it is, however, possible that the utilization of pantothenic acid was inhibited by the isoniazid.

Summary. 1. Administering 50-75 mg/kg of isoniazid 3 times a week to the guinea pig produces a transient peripheral neuropathy characterized by initial hyperreflexia and hyperesthesia particularly evident in limb joints. An extensive muscle flaccidity of 3-4 hours duration also occurs during this reaction to isoniazid. 2. Addition of 1.2 g/kg of ethanol to the isoniazid injection significantly increases incidence of the toxic response to isoniazid. 3. The response is prevented by daily administration of 20 mg/kg of calcium pantothenate but is not prevented by daily administration of 20 mg/kg of pyridoxal HCl.

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Peroxidase and Catalase in Saliva.*† (23236)

John F. Nickerson, Frederick W. Kraus, and William I. Perry
(Introduced by T. F. Paine, Jr.)
Department of Microbiology, University of Alabama Medical Center and Veterans Administration Hospital, Birmingham.

Earlier work on peroxidase in saliva was qualitative with conflicting reports as to the source and the peroxidatic or oxidatic nature of the enzyme(1-5). Eggers-Lura(6) demonstrated presence of peroxidase in the parotid secretion and whole saliva. He noted the high peroxidase activity of the salivary sediment and suggested that this activity might be due to presence of leukocytes. Mosimann and Sumner(7) reported a strong peroxidase activity in whole saliva and salivary sediment. Catalase in saliva has received incidental mention by a number of investigators but only a few specific studies have been made. Deakins(8,9) investigated the effect of storage on catalase in saliva and the catalase activity of 14 samples of whole saliva. Eggers-Lura(6) claimed that catalase is present in the parotid gland secretion.

The present study extends these investigations and describes activities, properties, and sources of peroxidase and catalase in saliva.

Methods. For peroxidase determination the technic proposed by Sumner and Gjessing (10) was utilized unchanged except for a modification in final H_2O_2 concentration and for omission of the ether filtration. One ml of saliva was used in all determinations and the activity was calculated as milligrams of purpurogallin formed per ml of enzyme source. The color development was read in a Klett Summerson photoelectric colorimeter and compared with a standard curve. Pyrogallol was of reagent grade quality, and fresh solutions were prepared daily and maintained at 4°C. The cytochemical nickle-ammonium-sulfate benzidine stain for peroxidase was used essentially as it was described(11) except for the change in final hydrogen peroxide concentration from 0.015% to 0.0025%. This change shortened the staining time from 2 min to 30 sec. The counterstain employed was freshly filtered carbol-fuchsin. The guaiacol stain for peroxidase consisted of 99.5 ml of a 1.0% guaiacol solution and 0.5 ml of 0.5% H_2O_2. The staining time was 30 sec and the counterstain employed was carbol-fuchsin. Indicators used for determination of bacterial peroxidase were 2-7-diaminofluorene (Eastern Chemical Corp.) and benzidine. The reaction mixture consisted of 3.0 ml of a saturated solution of either indicator, 1.0 ml of saliva and 1.0 ml of H_2O_2. The final concentration of H_2O_2 ranged from 0.001% to 1.0%. After 4 hr at room temperature, tubes were read visually and recorded on a relative basis. Catalase activity was measured by the method of Feinstein(12). Activity was recorded as meq of perborate destroyed per ml of enzyme source. On the basis of Feinstein's data the relation of perborate units to the classical *Kat. f.* would be approximately 1:100. Duplicate samples and appropriate controls were used in all determinations. Whole saliva was collected by paraffin stimulation several hours after a meal. The parotid secretion was collected with a Curby cap(13) and the submaxillary secretion was collected with Schneyer's segregator(14). One percent acetic acid applied to the dorsum of the tongue was used for stimulation in both instances. Contamination of secretions by whole saliva was eliminated by thorough rinsing of the mouth and drying of the mucosa adjacent to the salivary ducts before placing the appliances. Whole saliva and glandular secretions were refrigerated at 4°C immediately after collection, and activity determinations were performed within 2 hrs.

Results. Peroxidase. Average peroxidase activity of whole saliva at 11:00 a.m. was 1.198 ± 0.3004 mg of purpurogallin formed

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† Presented in part at meeting of Internat. Assn. for Dental Research, St. Louis, Mo., Mar. 1956.

TABLE I. Mean Effect of Inhibitors on Peroxidase Activity in 10 Samples of Whole Saliva and Glandular Secretions.

Compound	Molarity 1 ×	% Inhibition
Sodium fluoride	10^{-2}	42
"	10^{-1}	85
azide	10^{-3}	92
"	10^{-2}	100
Potassium cyanide	10^{-3}	85
" "	10^{-2}	100

per ml(15). When this activity was calculated on a dry weight basis, it was found that whole saliva had a P.Z.[‡] number ranging from 0.072 to 0.121. (For this calculation average dry weight of whole saliva was taken to be 12.4 mg/ml(16)). Peroxidase activity in whole saliva and glandular secretions was inhibited by several compounds (Table I). Temperature inhibition of peroxidase activity was determined by heating saliva for 10 min. When using the pyrogallol method the activity of both whole saliva and glandular secretions was rapidly lost above 80°C with total inhibition at 90°C.

Optimal substrate concentration for the peroxidases in saliva was determined by addition of graded concentrations of standardized H_2O_2 to the reaction mixture. Eleven samples of whole saliva and 2 samples of glandular secretion (1 parotid, 1 submaxillary) were used in the determination. The relationship of enzyme activity to optimum substrate concentration followed mass action principles in that samples with higher peroxidase activity had a higher optimum substrate concentration. Fig. 1 summarizes the data obtained with the 11 samples of whole saliva. Average maximum peroxidase activity for both whole saliva and glandular secretions was observed at a final concentration of 50 μg of H_2O_2 per ml. This concentration was used throughout the present study. It corresponds to the normal range of peroxidatic activity(15).

This is the optimum substrate concentration for salivary peroxidase in the presence of catalase. Although pyrogallol greatly inhibits its catalase, a residual activity remains(17). Apparently the residual activity did not alter the results since the glandular secretions which are catalase free had the same optimum substrate concentration as whole saliva.

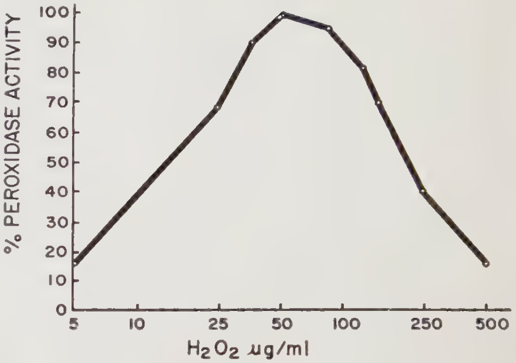

FIG. 1. Mean optimum substrate conc. for peroxidase in whole saliva.

Previous investigators(6,7) have reported that a portion of the peroxidase activity can be recovered in the sediment. When whole saliva from 25 subjects and 8 samples of glandular secretion (4 parotid, 4 submaxillary) were centrifuged at 1400 x Gravity for 20 min at a temperature of 8°C, the supernate of whole saliva was on an average 9.9 ± 6.7% less active than the uncentrifuged control. Glandular secretions showed no loss on centrifugation. The 2 most likely sources of activity in salivary sediment are leukocytic (myeloperoxidase(18)) and bacterial peroxidase.

Histochemical examination of the whole saliva sediment (Fig. 2) revealed a large num-

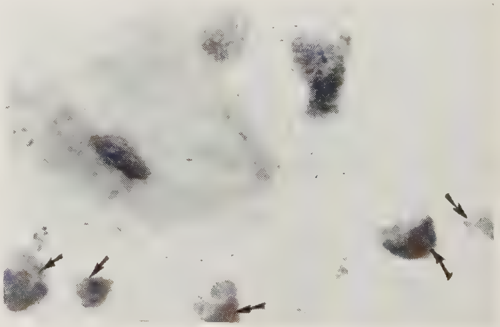

FIG. 2. Saliva sediment stained with NAS benzidine and carbol fuchsin. Epithelia and bacteria are stained red. Arrows point to blue peroxidase-positive granules in the cytoplasm of polymorphonuclear leukocytes. Original magnification 970 ×.

[‡] P.Z. or purpurogallin number is number of mg purpurogallin formed/mg of enzyme in 5 min at 20°C.

ber of peroxidase positive cells which on the basis of morphology and of intensity of the staining reaction were identified as polymorphonuclear leukocytes mainly of the neutrophilic series. They were seen infrequently in smears prepared from glandular secretions.

Bacterial peroxidase has been reported in a variety of organisms, particularly the streptococci. Bacterial peroxidase is said to be heat stable(19), while peroxidase in saliva was heat labile. Since pyrogallol is a poor indicator of bacterial peroxidase activity, 2-7-diaminofluorene and benzidine(20) were substituted in the search for a heat stable peroxidase. With these indicators a slight residual activity was detected after boiling saliva for 15 min. This activity was present not only in whole saliva but also in the glandular secretions. The secretions are relatively bacteria free and this fact suggested that not bacteria but traces of hemoglobin or its derivatives (pseudo-peroxidases) were the source of this weak heat stable peroxidase.

The activities of the 2 major glandular secretions were compared with the peroxidase activity of whole saliva by sampling whole saliva, parotid secretion, and submaxillary secretion as closely together in time as possible. The time required for collection was usually 20-30 min. The activity of the secretions was calculated in percentage of the activity of the corresponding whole saliva. Eighteen series were determined. The parotid secretion averaged 125.1 ± 19.6% and the submaxillary secretion averaged 86.8 ± 15.3%. The lower proportionate activity of the latter may be due to a greater content of inert matter, *e.g.* mucin.

Catalase. Average catalase activity of whole saliva at 11:00 a.m. was found to be 0.169 ± 0.0603 meq of perborate destroyed per ml(15). On a dry weight basis this was approximately 0.0137 meq of perborate destroyed per mg.

Relative catalase activity of centrifuged samples of whole saliva from 25 subjects was determined as for peroxidase. The average loss of catalase activity by whole saliva was 36.6 ± 16.0%. Catalase determinations in 40 samples of glandular secretion gave negative results with exception of one specimen contaminated by whole saliva.

Discussion. The source of *peroxidase* in saliva is predominantly glandular and a smaller amount of activity is contributed by the leukocytes. The percentage determined by centrifugation can only be taken as an approximation since it is possible that a large number of leukocytes have degenerated and released varying amounts of peroxidase into solution. This portion of leukocytic peroxidase would be recovered in the supernate. In addition, activity of the supernate would be increased relatively, on a unit volume basis, by sedimentation of inert material such as large masses of epithelia, bacteria and food residue which are devoid of peroxidase.

Preliminary biochemical and histochemical determinations of peroxidase in the human submaxillary gland indicate that the serous acinar cells of this gland are a rich source of tissue specific animal peroxidase and that they actively secrete it. This probably also holds true for the parotid gland. Plasma levels of peroxidase for all practical purposes can be considered to be non-existent.

Bacterial peroxidase is thought not to contribute to the peroxidase activity of saliva. The action of boiled saliva on benzidine and 2-7-diaminofluorene in the presence of H_2O_2 was slight and probably due to hemoglobin or its equally non-specific derivatives. Also, these indicators are highly sensitive and may give false positives(21,22) with various non-enzymatic materials. Hemoglobin has peroxidase-like action but is not specific for any one tissue of the body and is a constant source of contamination in studies of tissue specific peroxidases in animals. Hemoglobin was eliminated as a major source of peroxidase activity in saliva on the basis of the following evidence: The P.Z. of saliva on a dry weight basis has in some cases twice the peroxidase activity of crystalline oxyhemoglobin as determined by Bancroft and Elliott(17). The peroxidase activity of hemoglobin is relatively heat stable whereas the activity in saliva is heat labile. Microscopic examination of saliva utilizing histochemical stains for peroxidase failed to disclose any intact erythrocytes.

Absence of detectable hemoglobin and of

intact erythrocytes probably excludes the latter from consideration as a source of *catalase* in the healthy mouth. Since the glandular secretions are devoid of catalase it is presumed that the enzyme is primarily bacterial in origin. The fact that only 1/5 to 1/2 of the activity could be centrifuged out of solution may be explained by bacteria remaining in the supernate and by accumulated extracellular portion of the enzyme which would also remain in the supernate. Some authors (23,24) have reported that leukocytes contain catalase in the same quantity as erythrocytes. Agner(25) utilizing improved technics concluded that leukocytes are free of catalase.

Summary. 1. Peroxidase in normal saliva is predominantly of glandular origin with a smaller amount of activity contributed by the leukocytes. Hemoglobin may be present in trace amounts. Although saliva contains large numbers of streptococci said to contain bacterial peroxidase, the latter could not be demonstrated. 2. Catalase was absent from the glandular secretions in a large series of determinations and its origin in normal saliva is presumed to be bacterial. Leukocytes and erythrocytes probably contribute little if any catalase to normal saliva.

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"Spleen Protection" in Gamma- and Neutron-Irradiated Mice.* (23237)

HOWARD H. VOGEL, JR., JOHN W. CLARK, DONN L. JORDAN, NICHOLAS BINK, AND RICHARD R. BARHORST (Introduced by T. N. Tahmisian)
Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill.

Several investigators have confirmed the protective effects of spleen-shielding, injections of spleen homogenates, or infusions of bone marrow in X-irradiated mammals. Since there is evidence that fission neutrons and Co^{60} gamma rays may kill mice through different modes of mortality(1), it has been suggested that the earlier neutron death is largely attributable to effects on the intestine while the later X- or gamma-ray mortality peak is primarily associated with destruction of the hematopoietic system. It seemed of interest, therefore, to determine whether or not implanted spleens would protect neutron-irradiated mice. Since it was impracticable to attempt to shield the spleen from fission neutrons, attempts were made to utilize some of the other technics that have been developed. In preliminary experiments, several attempts were made to use Cole's technics(2) to protect neutron-irradiated mice with homogenates of spleens, but no protection was afforded. The experiments reported here were suggested by Jacobson's earlier work(3), in which spleens from newborn mice were implanted into the abdominal cavities of irradiated mice.

Methods. Four spleens from CF No. 1 mice (< 2 weeks old) were implanted into an ether-anesthetized, neutron-irradiated mouse, within 2 to 3 hours after irradiation. All exposures were carried out in the gamma-neutron radiation chamber(4) using fission neutrons at the CP-3′ and CP-5 Reactors at Argonne National Laboratory. Neutron exposures were for 2 hours or less; dose rates varied from 3-10 rads/min. The irradiated mice were CF No. 1 females 6-8 weeks of age. Half of each series of irradiated animals received spleens and half served as sham-operated controls, in which the body cavity was opened, a forceps inserted momentarily, and the incision closed. A third group of animals was randomly selected as unirradiated controls. There were 20 to 30 mice in each of these 3 groups.

Results. In Fig. 1, the results of 3 neutron experiments are summarized. In each case, the upper figure compares the average body weights of the 3 groups of mice, while the lower graph compares the cumulative mortality of the mice receiving spleens after irradiation with that of the sham-operated, neutron-irradiated group in the same experiment. There was no significant difference in body weight loss between the spleen-implanted and sham-operated groups. The cumulative mortality of the spleen-implanted groups was almost identical with that of the sham-operated groups at 30 days, whether the neutron dose was sufficiently high to kill 25%, 60%, or 80% of the mice. These data suggest that *spleen implantation gave no protection to the mice irradiated with fission neutrons.*

In all cases of death, autopsies were performed. Several implanted spleens were usually recovered from the body cavities of the irradiated mice. They were found in all stages from apparent normality to complete destruction, as judged by gross inspection. The implants were usually firmly attached to peritoneal surfaces.

To test the method, 2 similar experiments were carried out following 940-950 r Co^{60} gamma irradiation (exposure approximately 1 hour, dose rate ~ 16 r/min.) Both weight loss and mortality appeared much later (end of the second week) after gamma irradiation than after comparable fission neutron exposures (Fig. 2). In both of these gamma ray experiments, the 30-day mortality data indicate that the spleen implants definitely conferred some protection. This is also evident in the lack of weight loss from the 10th to the 15th days in the spleen-implanted mice.

In view of the fact that the protection after

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* Work performed under auspices of U. S. Atomic Energy Commission.

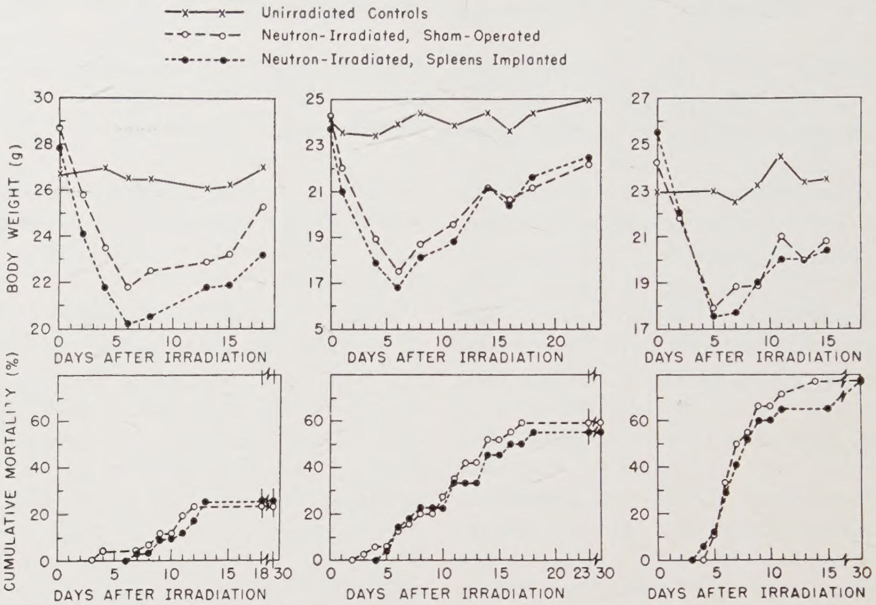

FIG. 1. Effects of spleen implantation on body weight and cumulative mortality of neutron-irradiated mice.

gamma irradiation was most evident at the end of the second week after exposure, it is perhaps not surprising that implantation of spleens did not protect neutron-irradiated mice during the early period, when mortality was high. By this time most of the neutron-irradiated mice were dead, presumably from a different mechanism of mortality. It is conceivable that the use of an antibiotic such as streptomycin, which can protect neutron-irradiated mice for at least 10 or 11 days after exposure(5), would keep a sufficient number

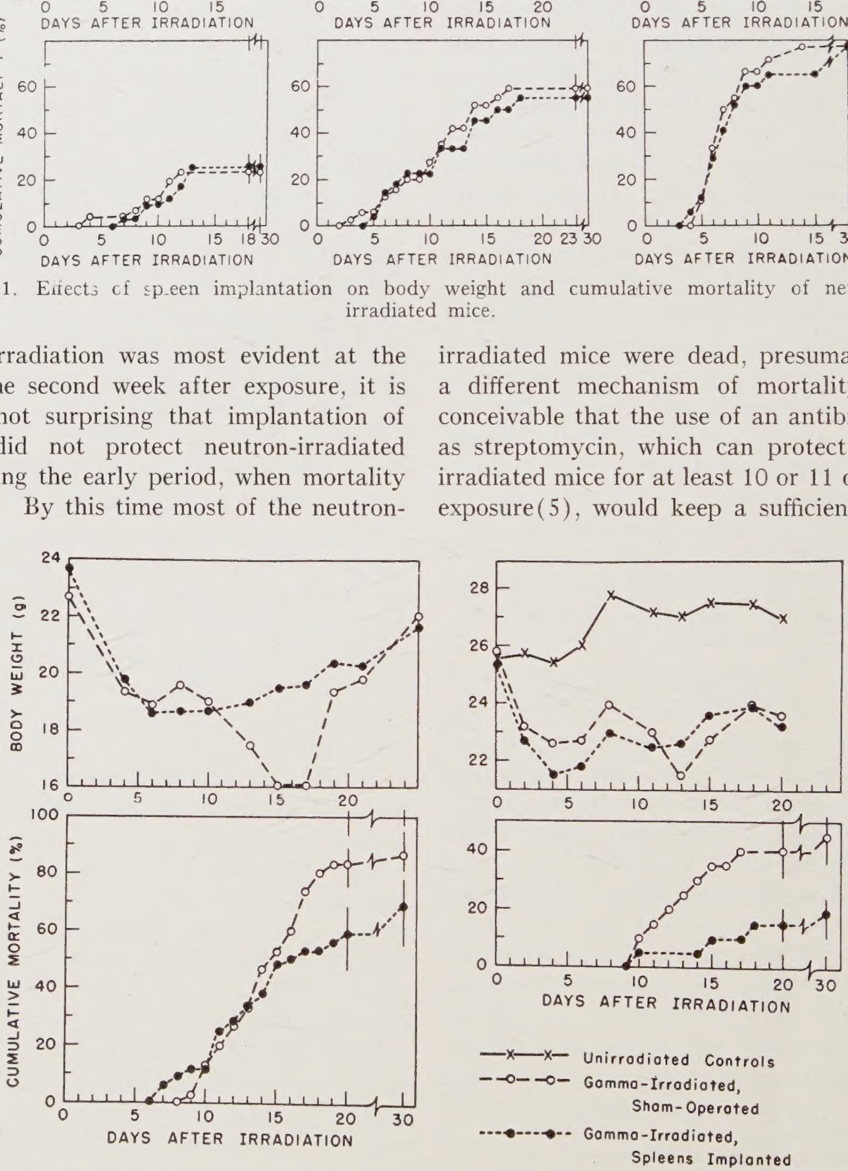

FIG. 2. Effects of spleen implantation on body weight and cumulative mortality of gamma-irradiated mice.

of mice alive so that the spleen implants could then help prevent the later death, associated with destruction of the blood-forming elements. Such an experiment is now being carried out.

Randolph and Congdon(6) have exposed RF male mice to total-body irradiation with 14 Mev neutrons generated by a Cockcroft-Walton linear accelerator. Preliminary results indicate that post-irradiation treatment with isologous bone marrow cells (intravenous injection) increased the 30-day survival. These mice were exposed to 650 rads of 14 Mev neutrons. Only 20% of the irradiated animals died during the first 8 days after exposure. Following a comparable dose (250 rads) of fission neutrons (mean energy, ~ 1 Mev), 86% of the irradiated mice (67/78) died from days 3 through 8 (7). These results indicate that fast neutrons of different energies may produce different modes of mortality, and that the early 4 to 10 day death characteristic of fission neutrons(7) is not so evident following 14 Mev neutrons.

Conger(8) has recently shown that for *Tradescantia* chromosomal aberrations there is a consistent increase in relative biological effectiveness (RBE) with linear energy transfer (LET), which is almost linear from the X-ray to the neutron energy range *and within the neutron energy range.* Since the linear energy transfer (Kev lost per micron) is approximately 3 times higher for 1 Mev than for 14 Mev neutrons, it seems probable that differences may occur in biological effects from neutrons of such different energy ranges.

It is significant that bone marrow treatment of the mice did not prevent death during the first week after irradiation, the period of high mortality following fission neutrons. It seems probable from these data that radiation with fast neutrons of different energies may give different results after treatment with blood-forming cells. This hypothesis is further substantiated by some recent work by Cole and Ellis(9) on the effect of bone marrow or spleen injections in LAF_1 mice after fast neutron irradiation. Mice were exposed to neutrons (2 Mev and 8 Mev) at the Berkeley 60-inch cyclotron. They received a single postirradiation intravenous injection of fresh isologous bone marrow (10^6 nucleated cells) or a single i.p. injection of isologous infant spleen homogenate (45 mg/mouse). Little or no protection of the neutron-irradiated mice (425-510 rep) was reported either for 30-day survival or for mean survival time. These data are in marked contrast to results observed with lethally X-irradiated mice.

Cole's work with intravenous bone marrow and our results with spleen implants confirm the lack of protection of mice exposed to fast neutrons, at least from 1 to 8 Mev. They add to the increasing evidence that the early death following fission neutrons is not a hematological death.

Summary. 1. Young adult female CF No. 1 mice were irradiated with single whole-body exposures of fission neutrons with dose range sufficient to kill 20 to 90% in 30 days. Four spleens from 2-week-old mice were implanted into body cavities of half this group; the remaining mice served as sham-operated controls. 2. Spleens gave no obvious protection to these neutron-irradiated mice: There was no significant difference in either body weight loss or in 30-day mortality between spleen-implanted and sham-operated groups. 3. Implanted spleens provided some protection to similar mice exposed to single doses of Co^{60} γ-rays in the 30-day lethal range. 4. Failure of implanted spleens to protect neutron-irradiated mice may be correlated with early (4-10 day) mode of mortality following single doses of fission neutrons. 5. It seems probable that radiation with fast neutrons of different energies may give different results after treatment with blood-forming cells.

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